

PHYSICOCHEMICAL STUDIES ON STARCH  
AND ITS COMPONENTS.

by

Samuel L. MacKenzie, B.Sc.

Thesis submitted for the degree of

Doctor of Philosophy

in the

Faculty of Science

University of Edinburgh.

August, 1964.





# ABSTRACT OF THESIS

Name of Candidate Samuel Leonard MacKenzie,  
Address [REDACTED]  
Degree Doctor of Philosophy Date August, 1964.  
Title of Thesis Physico-chemical Studies on Starch and its Components.

---

After a brief survey of current concepts in the chemistry of starch and its components, the physical and enzymic techniques used in this work have been discussed in detail.

Potato starch has been fractionated by a chloral hydrate method; the products obtained were comparable to those obtained by more conventional methods of fractionation. The efficiency of polar complexing agents for separating amylose has been investigated; n-butanol and n-amyl alcohol have been found to be preferable. Hydrophobic complexing agents have also been used to fractionate starch. Pure amylopectin fractions were readily obtained and amyloses of a high degree of purity were obtained on recrystallisation.

The effect of a commercial extraction procedure on the fine structure of maize starch has been investigated. Limited degradation of the amylose component was found to occur. The fine structure of the amylopectin from high-amylose-content maize starch has been examined in detail; the apparent long chain-length of amylopectin has been shown to be due to contamination by short-chain amylose.

An investigation of the starch from the fruit of the potato showed that the unusually prominent granular markings were not coincident with gross changes in the granular structure.

A study of the properties of starches from the growing potato tuber showed that the gross properties both of starch and the components are a function of granule size. Profound changes /

*Use other side if necessary.*



changes in the fine-structure of the components also take place. The amylose component was shown, by viscosity and sedimentation measurements, to increase in molecular size; the  $\alpha$ -amylolysis limit, however, decreased with increase in granule size. The molecular weight of the amylopectin also increased and measurements of chain-lengths and  $\alpha$ -amylolysis limits indicated that the structure was becoming increasingly branched. Current theories of the biosynthesis of starch have been reviewed and discussed in terms of the results obtained.

High-energy electrons, administered in various doses have been shown to degrade potato starch and modify the structure of the components.



## C O N T E N T S.

	Page
<u>SECTION 1</u> Introduction.	1
<u>SECTION 2</u> Experimental Methods.	8
(a)    General Methods.	
(1) Estimation of percentage starch in plant material.	8
(2) Extraction and purification of starch.	9
(3) Estimation of percentage nitrogen.	9
(4) Gelatinisation temperature.	10
(5) Granule pretreatment with liquid ammonia.	11
(6) Estimation of reducing sugar.	12
(7) Enzymic degradation.	15
(b)    Periodate oxidation.	19
(c)    Phosphorus estimation.	23
(d)    Potentiometric iodine titration	28
(e)    Sedimentation	33
(f)    Viscosity	39
<u>SECTION 3</u> Studies on the Fractionation of Potato Starch.	
Introduction	46
I.    Chloral hydrate fractionation	50
II.    Fractionation by polar precipitants	56
III.    Fractionation by non-polar precipitants	66



## C O N T E N T S (Continued)

	Page
<u>SECTION 4</u> Studies on Maize Starch.	
I.    A Comparison of the Properties of Commercial and Laboratory-Extracted Maize Starches.	73
II.   Studies on High Amylose-Content Maize Starch.	81
 <u>SECTION 5</u> An Investigation of the Starch from the Fruit of the Potato, <u>Solanum tuberosum</u> .	
(a)   Introduction.	97
(b)   Experimental.	102
(c)   Results and Discussion.	106
 <u>SECTION 6</u> Biosynthesis of Starch: Studies on the Growth of the Starch Components.	
I.    A Study of the Properties of Starches from the Growing Potato Tuber.	
(a) Introduction.	114
(b) Experimental.	130
(c) Results and Discussion.	134
II.   A Study of the Properties of Starches from Granules of Different Sizes.	151
General Discussion.	157
 <u>SECTION 7</u> The Effect of High-Energy Electrons on Potato Starch and its Components.	
(a)   Introduction.	162
(b)   Experimental.	165
(c)   Results and Discussion.	167
 <u>SUMMARY</u>	177
 <u>BIBLIOGRAPHY</u>	179



## P R E F A C E.

Throughout the period of this research, Dr. C.T. Greenwood has been a constant source of advice and encouragement and I wish to take this opportunity to express my gratitude to him.

The work described in Sections 5 and 7 has been published in conjunction with Dr. Greenwood and reprints of these papers are inserted at the end of this thesis.

I wish to thank Professors Sir Edmund Hirst and T.L. Cottrell for the provision of laboratory facilities and the Corn Industries Research Foundation for a maintenance grant.

I also wish to thank Miss Tarrant for taking the photomicrographs inserted in this thesis.

The University Chemistry Department,  
The King's Buildings,  
EDINBURGH, 9.



SECTION 1.

INTRODUCTION.



## INTRODUCTION.

Starch is the most abundant reserve carbohydrate of the plant world and is found in most higher plants as well as in some microorganisms. Despite the importance of starch and despite the great deal of attention paid to the substance, it is only in comparatively recent years that any clear understanding of its structure has been attained.

Starch occurs in the plant in the form of granules varying in shape and from 1-150 $\mu$  in diameter. Each plant species forms granules of a somewhat variable, but species-specific form and granules from different plants may be recognised microscopically. The granule consists of an inner nucleus, termed the hilum, around which are deposited concentric layers. The appearance of these layers is due to discontinuities in the index of refraction of the deposited material. Within each layer the index of refraction changes continuously from a higher to a lower level but, at the border of each new layer, the index of refraction jumps suddenly to a higher value. These layers may be quite conspicuous; for instance, the starch granules from the berry of the potato plant have unusually prominent lamellar markings. The factors governing those pronounced markings are not yet known.

There has been considerable speculation regarding the nature of the molecular architecture within the granule. The concept which is most widely accepted at the present time was formulated /



formulated by Meyer in 1942. According to this theory the starch molecules are arranged in a radial fashion. Where linear sections of the starch molecules run parallel to one another, hydrogen-bonding forces pull the chains together into associated crystalline bundles or "micelles" which are responsible for binding the granule together. In the spaces between the micelles the chains and branches are more disordered and hence less densely packed. A radial orientation of the starch molecules, as postulated by Meyer, is necessary to account for the phenomenon of birefringence exhibited by the granules; many of the swelling and leaching properties of the starch granule can also be explained on the basis of a "micellar" structure.

While it is now generally accepted by botanists that starch is synthesised only by the plastids of cells, there has been much speculation regarding the enzyme system or systems responsible. Many theories have also been put forward for the mode of deposition of starch in the granule. However, little is known of the manner in which the structure of starch and its components varies in the growing plant.

Perhaps the most fundamental advance in the whole field of starch chemistry was the realisation that the starch granule is composed of, at least, two chemically and physically distinguishable components. Although the concept of the heterogeneity of starch had for long found wide acceptance, it was not until 1942 that efforts to achieve a quantitative separation of the components were successful. A considerable amount of research on this topic has /



has since been carried out, but many aspects of fractionation are still not clearly understood.

Most native starches contain ca 20% of an essentially linear polymer, termed amylose, consisting of chains of D-glucopyranose units joined by  $\alpha$  -1:4 glycosidic linkages. The major component of starch - amylopectin - is highly branched and also contains chains of D-glucopyranose units. While the linkages in the linear portions of amylopectin are also  $\alpha$  -1:4 glycosidic ones, the many branch points take the form of  $\alpha$  -1:6 linkages.

The essentially linear nature of amylose was first suggested by Meyer et al (1940), when the osmotically-determined molecular weights of potato and maize amyloses were found to agree with the chain-lengths of 200-300 D-glucose residues as determined by end-group analyses. Periodate oxidation has also been applied to amylose, but, although this method has been more satisfactory than methylation, more recent work suggesting a chain-length for amylose of several thousand units, renders its accuracy rather questionable. In fact physical methods such as viscosity determinations have largely replaced the classical methods of examination of amylose.

Enzymic studies have led to the belief that amylose may not be completely linear. For instance, pure  $\beta$ -amylase which, operating exclusively on non-reducing terminal units, exerts a hydrolytic action on  $\alpha$  -1:4 linkages, does not completely degrade amylose molecules to maltose. Although it is now generally accepted that there are present, in some amylose molecules, modifications which present a barrier /



barrier to  $\beta$ -amylolysis, no widespread agreement exists as to the nature of these modifications. It has been suggested (Greenwood, 1960) that amylose molecules with a barrier to  $\beta$ -amylolysis may well contain branches while some workers (Kjølberg and Manners, 1963) indicated that the branches may take the form of  $\alpha$ -1:6 linkages.

Methylation and periodate oxidation studies on a number of amylopectins have indicated that the average length of unit chain may vary from 18-27 anhydroglucose units. The presence of  $\alpha$ -1:6 branch points was shown by the isolation of isomaltose from various amylopectins following enzymic hydrolysis (Montgomery et al, 1949). Final proof of the nature of the branch points, was obtained by Thomson and Wolfrom (1951) who discovered isomaltose and panose in the partial acid hydrolysis of waxy maize amylopectin. Periodate oxidation has also confirmed that the majority of branch linkages in amylopectin are  $\alpha$ -1:6 but there are indications, derived from the isolation of glucose from periodate-oxidised starch residues, that 1:3 or 1:2 linkages may also be present (Hamilton and Smith, 1956). More recent work (Manners and Mercer, 1963) however, has failed to confirm these findings.

In considering a structure consisting of a number of short chains of  $\alpha$ -1:4 linked anhydroglucose units joined to each other by  $\alpha$ -1:6 branch points, there arises the question of how these short chains are arranged in the molecule. The three principal /



structures proposed for amylopectin are shown in Fig. 1. 1. The laminated structure proposed by Haworth et al (1937) is the simplest consistent with methylation studies but does not give a complete representation of the molecule. Staundinger and Husemann's (1937) "herring-bone" structure was derived by comparison of the viscosity of amylopectin with that of cellulose of the same molecular weight. A more accurate representation of the amylopectin molecule was achieved only as a result of enzymic studies. Meyer and Bernfeld (1940) showed that  $\beta$ -amylase removed only ca 50% of the molecule leaving a high molecular weight residue. As a result, they proposed a ramified "tree-like" structure.

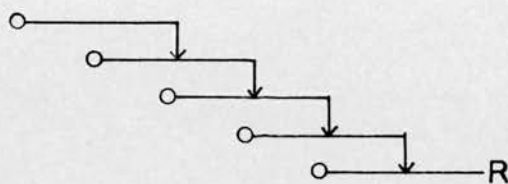
Myrbäck and Sillen (1949) showed that the three structures postulated for amylopectin merely contain different arrangements of the same basic linear chains. The chains were later termed A-, B- and C-chains by Peat et al (1952). The A-chain is linked to the rest of the molecule only through its reducing end-group; the B-chain, in addition to being linked as an A-chain, is also substituted through the C6-hydroxyl group in one or more of its constituent glucose units; the C-chain carries the only reducing end-group in the molecule. In structure (a) (Fig. 1. 1) there is only one A-chain, in (b) no B-chains and (c) approximately equal numbers of A- and B-chains. By the successive use of  $\beta$ -amylase and R-enzyme, Peat et al (1952) concluded that the Meyer structure is the most probable representation of the amylopectin molecule.

While the majority of starches contain a minor proportion of linear material and a major proportion of branched material, the starches /

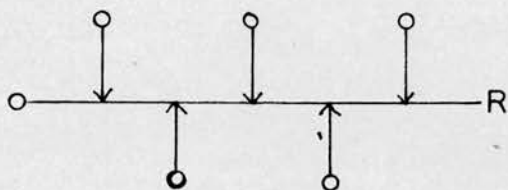


FIG. 1.1

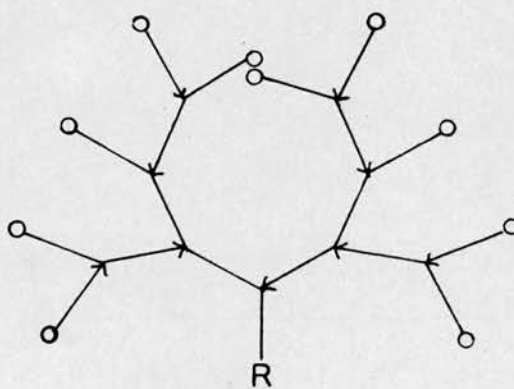
(a) Haworth



(b) Staudinger



(c) Meyer



R Reducing end group  
 ○ Non reducing end group  
 ↓  $\alpha$ -1:6 link



starches obtained from many cereal sources present a wider spectrum with regard to the ratio of the two components. For example, the starches of the waxy cereals are composed entirely of amylopectin. At the other extreme, maize starches containing ca 80% of amylose have been obtained as a result of developing knowledge of the genetic factors governing the amounts of the two components. Cereal starches differ from tuber starches in that the granules give the so-called A-type X-ray diffraction pattern; in addition, pretreatment is generally required before cereal starch granules can be sufficiently solubilised to allow effective fractionation. Despite the large amount of work carried out on maize starches, many problems related to their fractionation and fine structure remain unsolved.

#### Outline of Thesis.

In this thesis some of the outstanding problems in the field of potato and maize starches are examined in detail. Following the survey, in Section 2, of the experimental methods used in this work, various methods of fractionating potato starch are then studied. Section 4 is devoted to a study of the differences between commercial and laboratory-extracted maize starches. In addition, the fine structure of the amylopectin from high amylose-content maize starches is considered. The unusually prominent lamellations on the granules obtained from the potato berry are investigated in Section 5 and a detailed study then follows of the changes taking place in the structure of the starch components during maturation of /



of the potato tuber. Finally, Section 7 deals with the effect of irradiation, by high-energy electrons, on the starch granule and its components.



SECTION 2.

EXPERIMENTAL METHODS.



(a) GENERAL METHODS.

(1) Estimation of Percentage Starch in Plant Material.

The starch content of plant material was estimated by extraction with perchloric acid (MacWilliam, Hall and Harris, 1956) followed by colorimetric estimation of the resultant extracts (Dubois et al, 1956).

A sample (200mg.) of the plant material was ground in a mortar with silver sand (200mg.) and then transferred to a centrifuge tube. After heating on a boiling water bath to gelatinise the starch, the tube was cooled in an ice bath and perchloric acid (6 ml; 72%) slowly added with constant stirring; any undissolved material was ground on the sides of the tube with the aid of the sand and a glass rod. After 20 minutes, the mixture was diluted to about 40 ml. with water and centrifuged. The clear supernatant liquid was transferred to a volumetric flask. The extraction procedure was repeated until on washing the residue with water, the washings gave no blue colour with a solution of iodine (0.2%) in potassium iodide (2%). The combined extracts and washings were made up to a standard volume and the carbohydrate content was estimated by the phenol-sulphuric acid method. Determinations were made in duplicate, giving results agreeing to  $\pm 5\%$ .

(2) /



(2) Extraction and Purification of Starch.

Starch was isolated from plant material by a mechanical process involving minimum degradation of the starch (Banks and Greenwood, 1959). In the case of potatoes the tubers were first thickly peeled, cut up into slices and minced, the resultant product being kept under mercuric chloride (0.01M) to inhibit enzymic activity. In the next stage, the material was further subdivided by extraction in a Blendor for 2 - 3 minutes. The pulp so obtained was filtered through muslin and the granules separated by repeated sedimentation in sodium chloride solution (0.1M). Contaminating protein was removed by shaking a saline suspension of the granules with toluene. After sedimenting through water several times to remove salt, the granules were stored in aqueous suspension at 0°C under toluene.

Prior to fractionation the granules were further purified by the removal of small amounts of contaminating fatty substances. This was achieved by stirring the granules under reflux in aqueous methanol (85%) with repeated changes of solvent. (Schoch, 1942).

(3) Estimation of Percentage Nitrogen.

The percentage nitrogen was obtained by the semi-micro Kjeldahl method. Determinations were made in duplicate.

(4)/

(4) Gelatinisation Temperatures.

The gelatinisation temperature is that temperature at which starch granules lose their birefringent properties, when heated in a suitable swelling medium, in this case an aqueous suspension. Since any starch sample contains a range of granule sizes and large granules are generally disrupted more easily than small granules, a gelatinisation range of 5 - 10°C is observed. Consequently, the temperatures of initiation and termination are often quoted (Schoch and Maywald, 1956). Alternatively the results may be represented by the average gelatinisation temperature, which corresponds to loss of birefringence by 50% of the granules.

The gelatinisation temperature was determined using a Kofler electrically heated microscope stage. A drop of starch suspension (0.1 - 0.2%) was placed on a microscope slide and surrounded by a ring of viscous oil. After carefully covering with a micro cover-glass so that no air bubbles were formed and the oil seal remained intact, the slide was placed on the hot stage and enclosed by a glass plate to prevent the formation of convection currents and provide uniform temperature. Suitable adjustment of a variable transformer gave a rate of temperature rise not exceeding 1°C per minute. Observations were made using a microscope fitted with Nicol prisms, first using normal light but on commencement of granular swelling polarised light was used to show the loss of birefringence.

Gelatinisation /



Gelatinisation curves were obtained by measuring the temperature at which various percentages of granules lost their birefringent properties. (e.g. Fig. 5. 3).

(5) Granule Pretreatment with Liquid Ammonia.

Several methods have been used for the pretreatment of starch, often with the aim of making the granules more easily soluble in water. For instance, it is practically impossible to achieve a proper aqueous dispersion of high amylose content granules without prior treatment. Pretreatment has also been employed to facilitate the formation of starch derivatives e.g. methylation. The overriding consideration in all the aspects of pretreatment is that the starch components undergo the minimum possible physical and chemical degradation. This condition is best fulfilled by the liquid-ammonia pretreatment method of Hodge, Montgomery and Hilbert (1948), which has been shown (Banks, Greenwood and Thomson, 1959) to cause no degradation in wheat, barley and potato starches as evidenced by the properties of the respective amyloses isolated from aqueous dispersions.

An alcohol suspension of starch (10 - 15g) was treated for 15 minutes in a Dewar vessel with liquid ammonia (100ml.) The mixture was poured into ethanol (500 ml.) and allowed to stand overnight to allow the ammonia to evaporate. The starch was then filtered and thoroughly washed with ethanol.

All starches were pretreated thus before dispersion and fractionation. Microscopic observation showed that after liquid ammonia treatment, the granules had lost their birefringent properties. The treatment had, therefore, destroyed the crystallinity of the granule.

(6) Estimation of Reducing Sugar.

An essential feature of most of the techniques used in this thesis, is a knowledge of the concentration of polysaccharide in solution. Amyloses were most often handled in the form of the butanol-complexes and since amylopectin solutions were always filtered the concentrations were most conveniently obtained by acid hydrolysis of an aliquot of the solution followed by an estimation of the reducing power. The method of Somogyi, (1945) is widely applied but is subject to disadvantages when employed in certain facets of starch chemistry, especially when maltose is being estimated in the presence of iodine-staining polysaccharide. This occurs in the determination of the  $\beta$ -amylolysis limits of amylopectin and amylose. The copper reagent, used in the Somogyi estimation, releases iodine which can react with the polysaccharide, resulting in error in the end point of the thiosulphate-iodine titration. The method used to circumvent this difficulty was that of Lampitt et al, (1955) involving the reduction of alkaline ferricyanide and subsequent oxidation by ceric sulphate. This method has the additional advantage that repeated /



repeated calibration of the reagent against polysaccharide is unnecessary since the calibration factor is directly proportional to the normality of the ceric sulphate.

#### Experimental.

Aliquots of the polysaccharide solution (3 x 1 ml; each containing up to 3 mg.) were measured into Quickfit boiling tubes, (neutralised if alkaline) and sulphuric acid (1 ml; 3N) added. (Pirt and Whelan, 1951). Hydrolysis was achieved by heating the stoppered tubes on a boiling water bath for two hours. After allowing the tubes to cool, the contents were neutralised with M potassium hydroxide using bromocresol-green as indicator. Subsequently, each solution was diluted to about 10 ml. with distilled water; sodium carbonate solution (2.5ml; 0.2M) and potassium ferricyanide solution (2.5ml; 0.05M) were added. After a further 15 minutes boiling, sulphuric acid (5ml; 5N) and xylene cyanol F.F. indicator (2 drops; 0.2%) were added to the cooled tubes and the whole titrated with ceric sulphate (0.01M). The titration end point was indicated by a colour change from "sage green" to "whisky yellow".

Omitting the hydrolysis stage, the above method was also used to estimate maltose as a product of  $\beta$ -amylolysis.

#### Calibration.

"Analar" soluble starch was dried by heating in vacuo at /

at 60° for several hours. Standard solutions were made up in dilute alkali and 1 ml. aliquots withdrawn for hydrolysis and subsequent estimation of the reducing power. One ml. of ceric sulphate (0.01N) was found to be equivalent to 0.293 mg. of starch.

The maltose calibration was carried out using standard solutions of maltose. The reducing power was estimated directly without hydrolysis. The calibration factor was found to be 1 ml. ceric sulphate (0.01N) = 0.430mg. maltose.

#### Phenol-Sulphuric Acid Method.

An alternative method to the above is that of Dubois et al., (1956), which depends on the fact that simple sugars, oligosaccharides, polysaccharides and their derivatives (including the methyl ethers) with free or potentially free reducing groups, give an orange-yellow colour when treated with phenol and concentrated sulphuric acid. The colour produced is permanent and under the proper conditions the method can be expected to be accurate to within  $\pm 2\%$ .

This type of estimation, however, is more susceptible to gross error than the alkaline-ferricyanide one, since scrupulous cleanliness of apparatus and extreme purity of reagents is demanded. The main advantages lie in the speed of estimation and in the fact that much smaller aliquots are required.

#### Experimental /



### Experimental.

Aliquots of the polysaccharide solution ( $3 \times \frac{1}{40}\text{ml}$ ) were measured into clean test tubes using a micro-pipette. Phenol solution (2ml; 5%) was added followed by the quick addition of concentrated sulphuric acid (6ml; "Analar" grade) from a burette. Likewise a blank solution was made up to contain solvent, phenol and sulphuric acid. After allowing 1 hour for the tubes to cool, the optical densities of the final solutions were determined using an Eel photoelectric colorimeter (Filter No. 623). The concentration of the polysaccharide was obtained by comparison with a calibration graph.

### Calibration.

Standard solutions of "Analar" soluble starch were used. Aliquots ( $\frac{1}{40}\text{ml}$ .) were withdrawn using a micro-pipette and the colour developed by the procedure outlined above.

### (7) Enzymic Degradation.

Much of the present knowledge of the fine structure of amylose and amylopectin has been elucidated as a result of studies using a wide range of enzymes. While the behaviour and specificity of the enzymes may not be fully understood, the nature of their action on the starch polymers has provided information which supplements that obtained by chemical and physical methods. Present attention is restricted /

restricted to the endo and exo amylases represented by  $\alpha$ - and  $\beta$ -amylase respectively.

$\beta$ -amylase hydrolyses amylose and the external chains of amylopectin to yield maltose. The enzyme attacks the non-reducing end units and degrades the molecule in a stepwise manner; it is capable of hydrolysing only 1 - 4 linkages and while the linkages are in the  $\alpha$ -configuration in the original polysaccharide, the reducing groups in the liberated maltose are in the  $\beta$ -configuration; so that inversion has occurred during the hydrolytic process.

$\beta$ -amylase was generally supposed to hydrolyse amylose completely to maltose; however, Peat and coworkers (1952), observed that the action of crystalline sweet-potato  $\beta$ -amylase, and highly purified soya-bean  $\beta$ -amylase on amylose at pH 4.8 ceased at ca 70% conversion into maltose. This incomplete hydrolysis of amylose is most adequately explained by the presence of some structural anomalies in the polysaccharide. Since, unpurified soya bean  $\beta$ -amylase preparations caused complete hydrolysis of amylose, the presence of a second enzyme in soya beans, designated Z-enzyme, was suggested. This contaminating enzyme is inhibited at pH 3.6 so that use of impure  $\beta$ -amylase at this pH will produce hydrolysis equivalent to use of the pure enzyme at pH 4.8. Z-enzyme has been shown (Banks, Greenwood and Jones, 1960) to cause a random hydrolytic cleavage of  $\alpha$ -1:4 bonds in amylose and /



and amylopectin. Since the concurrent action of Z-enzyme and  $\beta$ -amylase completely hydrolyses amylose to maltose, the purity of an amylose sample may be readily determined. If in the presence of Z enzyme, an amylose sample is not completely hydrolysed into maltose, then the amylose must contain contaminants, the amounts of which can be easily calculated.

Unlike  $\beta$ -amylase, the  $\alpha$  type enzyme acts in a more or less random manner on the  $\alpha$  -1:4 linkages in the substrate molecule.

#### Experimental.

The unit of  $\beta$ -amylase activity used was that defined by Hobson, Whelan and Peat (1950), i.e. the amount of enzyme which when incubated for 30 minutes at 35°C will liberate 1 mg. of maltose from a starch solution prepared as follows: starch solution (0.6%; 25ml.); acetate buffer (pH 4.6, 4ml.) and enzyme solution (1ml.). The enzyme concentration was adjusted so that 10-20 mgs. of maltose were produced during the period of incubation.

Digests were set up in 25 ml. "Quickfit" flasks and contained polysaccharide (10ml; of known concentration, ca 0.1%) buffer (4ml; see below) and enzyme solution. Incubation was at 36°C for a period of 24 hours. Liberated maltose was estimated by the alkaline ferricyanide method.

In the earlier stages of this work, crude  $\beta$ -amylase preparations were used and hence the digest pH was 3.6.

At /

At a later stage, however, a supply of pure  $\beta$ -amylase became available and pH 4.8 was used. At this latter pH the  $\beta$ -amylase is more active.



(b) PERIODATE OXIDATION.

The selective oxidation of  $\alpha$  -glycols by the periodate ion, giving two aldehyde groupings was first discovered by Malaprade in 1928. Since its discovery, the periodate reaction has been applied to a large variety of problems in all fields of organic chemistry. This reaction, however, has been used with most effect in the study of the carbohydrates, where the mild reaction conditions are particularly applicable. Another feature contributing to the wide usage of the periodate reaction and to its general displacement of methylation techniques, is its simplicity of application. Also, the aqueous solvent conditions lend themselves to use with the water-soluble carbohydrates.

When applied under proper conditions, the reaction is highly selective. Temperature, hydrogen ion concentration, absence of light, concentration of reactants and products as well as the use of periodic acid or one of its salts, all seem to influence the course of the reaction (Bobbitt, 1956). The rate of oxidation is greater for cis than for trans- $\alpha$ -glycols. For 1, 2, 3 - triols, the degradative oxidation results in the production of one mole of formic acid and the formation of two aldehydic groupings, two moles of periodate being reduced. This reaction is also valid for contiguous glycol groups in cyclic structures; the first application in this context was made by Jackson and Hudson, (1936). Thus /

Thus a chain of twenty  $\alpha$ -1:4 linked D-glucose units, in which the reducing end groups forms a 1-6 linkage to another similar chain, will consume twenty-one moles of periodate, since the non-reducing end group consumes two moles liberating one mole of formic acid. Estimation of the amount of formic acid released therefore enables the average length of unit chain to be determined. When dealing with an amylopectin, the relatively small amount of formic acid liberated from the single reducing end group can be neglected.

Two types of periodate oxidation may occur. The first, which may be termed selective, generally proceeds quickly and quantitatively giving, after a period of time, constant values for periodate uptake and for the formation of oxidation products. The second type, generally referred to as overoxidation, shows no marked levelling off at a maximum value. Reaction conditions are usually chosen to keep overoxidation to a minimum. It is worth noting that the occurrence of overoxidation may not give an exact guide to the completion of selective oxidation in the whole molecule. It is quite possible for a considerable number of glucose units in a polymer to be unattacked, while overoxidation of other units occurs. For example, it is known that in 1 - 2 diols with the hydroxyl groups locked in the trans position, periodate attack cannot occur, (Alexander et al, 1951); it is not inconceivable /



inconceivable in a high polymer such as amylopectin that glucose units may be held in just such a conformation thus preventing, or at least hindering, periodate attack. There are, moreover, numerous possibilities for inter and intra molecular linking in the dialdehydes produced on periodate oxidation.

To minimise the tendency of  $\alpha$ -1:4 glucosans to overoxidation, Brown et al (1948) used the sparingly soluble potassium meta-periodate at room temperature, the calculated excess of oxidant and of formic acid released being kept low. Potter and Hassid, (1948) introduced the use of a saline solution of sodium meta-periodate at 2°C for 25 hours. The method used in this work was a variation of the latter method by Greenwood and Thomson, (1962). Side reactions caused by the presence of 5% sodium chloride solution were avoided by the simple expedient of carrying out the oxidation in aqueous solution without added salt. The reagent blank was small and constant.

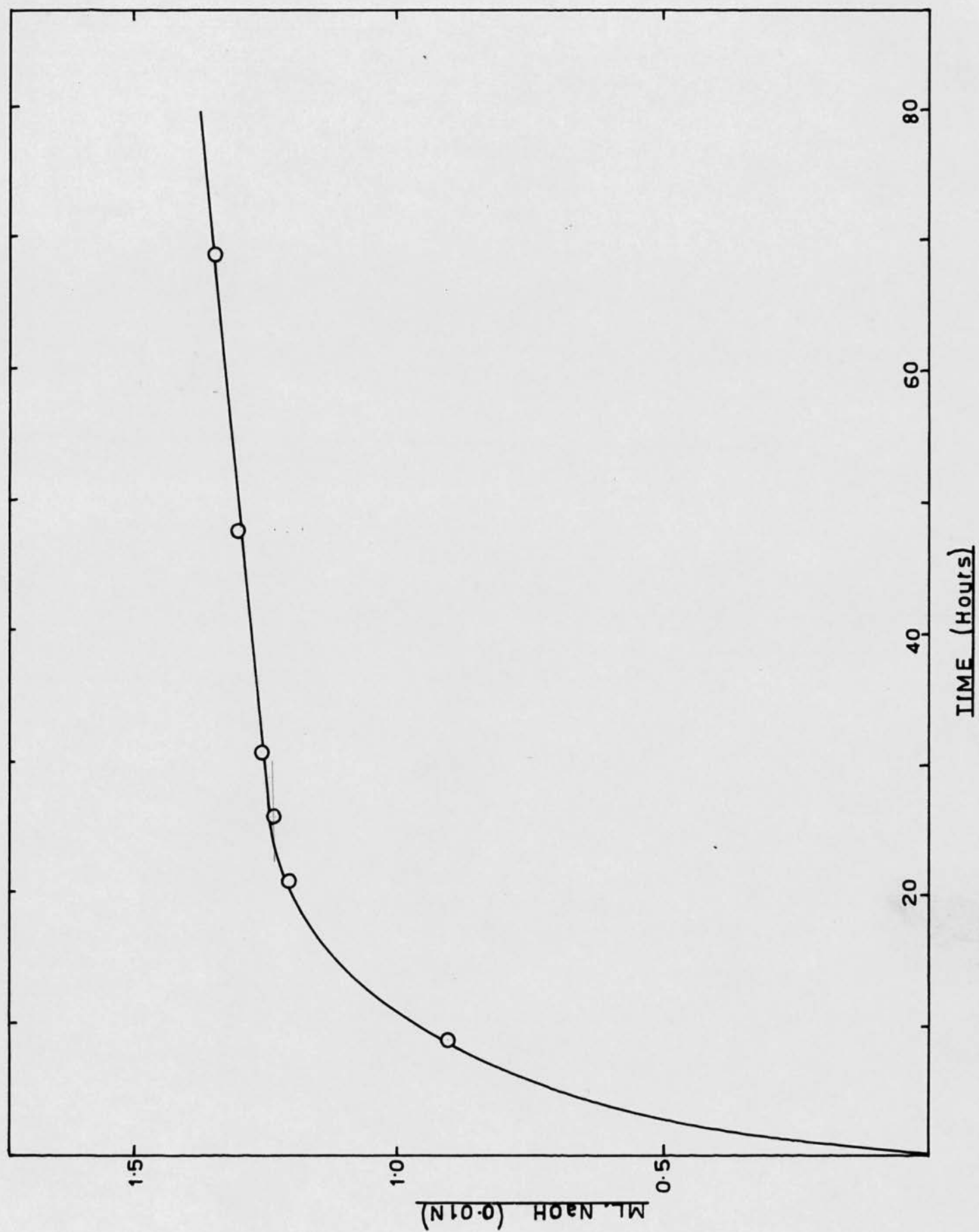
#### Experimental.

Polysaccharide (ca 200mg.) was dissolved in water (ca 70ml.; carbonate free) and cooled to 2°C. Sodium meta-periodate (20ml.; 0.58M) was added, the solution made up to 100ml. in a graduated flask and kept in the dark at 2°C. Portions, (20ml.) were removed for analysis. The excess periodate was destroyed by the addition of ethylene glycol (0.5ml) and the samples shaken in the dark at room temperature /

temperature for 30 minutes. The liberated formic acid was then titrated, in a nitrogen atmosphere, with sodium hydroxide (0.01M) on a Pye mains-operated pH meter. The titration end point was pH 6.25.

Under the conditions of Greenwood and Thomson, periodate uptake and formic acid release were considered to be constant after 25 hours and values of chain length were determined from titration values at this time. The periodate oxidations carried out in this thesis, however, were made on larger quantities of polysaccharide. It was then found that very limited overoxidation occurred (see Fig. 2. 1.). In view of the difficulty in correcting for this overoxidation, chain lengths were again determined from the titration figure after 25 hours. The values obtained will not, therefore, be absolute. However, it is thought that the error will be small and the method can thus be used with certainty as a tool for determining relative chain lengths.

FIG. 2.1.





(c) ESTIMATION OF PHOSPHORUS.

Most starches contain phosphorus, the amount present being considerably variable and dependent on the botanical source of the starch. In 1935, Posternak showed that most of the phosphorus in cereal starches was contained in the form of phosphatides, which could be extracted by hot water or methanol. On the other hand, the phosphorus in tuber starches was found to be linked to the polysaccharides in the ester form, being linked to carbon atom number six of a glucosyl unit.

(Schoch (1942) showed that most of the phosphorus present is joined to the amylopectin molecules. Further investigation by Posternak (1951) revealed that the phosphate groups in potato starch are not in the vicinity of reducing groups or of branching points. The amylose fraction contains only traces of phosphorus.

The determination of phosphorus in starch requires (1) the complete destruction of organic matter and (2) the quantitative conversion of phosphorus into the orthophosphate. The digestion of organic matter has been achieved by wet ashing with sulphuric and nitric acids, with sulphuric acid and hydrogen peroxide, with alkaline persulphate, or with perchloric acid. (Goodwin et al (1958) used chloric acid for the digestion of serum and serum extracts, while recently Saliman, (1964) developed a reagent containing hydriodic acid, calcium iodide, phenol and /

and acetic acid. The digestion procedure used is governed by the type of sample, the amount of phosphorus present and the method selected for the determination of the phosphate ion.

The method chosen here was that of Giesecking, Snider and Getz, (1935) - later examined more fully by Smith (1953) - employing perchloric acid in conjunction with nitric acid as a damping reactant. Following the action of cold, concentrated nitric acid, then the same reagent at rising temperatures, all readily oxidised organic material is destroyed. When the reaction of nitric acid is complete, the high boiling perchloric acid volatilises and displaces the boiling nitric acid. With its elimination, the 60% perchloric acid, upon further heating, concentrates slowly to the 72.5% water azeotrope. It is in this stepwise increase in oxidation potential that complex organic structures are broken up into fragmentation, degradation products. This accounts for the smooth, rapid oxidation without carbonization and without violent reaction. No loss of phosphorus through volatilisation occurs (Giesecking et al, 1935).

The orthophosphate may be estimated gravimetrically or volumetrically, but these methods are both time consuming and ill suited to the small quantities of phosphorus present in starch. The spectrophotometric method based on the molybdovanado-phosphoric acid complex has been extensively used /

used, (Gincotta, 1960) while the colorimetric method depending on the reduction of heteropolyphosphomolybdic acid is also particularly suitable.

The method used here is that of Fogg and Wilkinson (1958), in which the phosphomolybdic acid is reduced using ascorbic acid rather than the 1-amino-2 naphthol-4 sulphonic acid originally employed by Fiske and Subba Row (1925) and used frequently since (e.g. Bartlett, 1959). Colour development is rapid at the boiling point and, once developed, the molybdenum blue is extremely stable at room temperature.

#### Experimental.

Reagent: Ammonium molybdate - sulphuric acid solution.

Ammonium molybdate (10g.) was dissolved in distilled water and the solution diluted to 100 ml. To this solution was carefully added a solution of sulphuric acid (150 ml.; sp. grav. 1.84) in distilled water (150 ml.)

#### Digestion Procedure.

Dry polysaccharide (ca 75 mg. amylopectin; 100 mg. starch and 300 - 500 mg. amylose) was accurately weighed into a 25 ml. conical flask, mixed with concentrated nitric acid (0.25 ml.), gently heated on a hot plate till the initial reaction had subsided and then boiled till almost dry. The flask was removed from the hot plate and 0.5 ml. of a mixture of equal volumes of nitric (8 N) and perchloric acids (72%) added. The solution was then heated gently till all organic material had been removed. This stage was indicated by a colourless or slightly coloured solution /



solution. The flask was cooled and the sides rinsed with distilled water after which the contents were heated almost to dryness. Residual salts were dissolved by adding hydrochloric acid (0.5 ml; 2 N) and gently warming.

Colour development.

The acid solution obtained after the digestion procedure was washed into a 50 ml. beaker, diluted to about 40 ml. and ammonium molybdate sulphuric acid (4 ml.) solution added. After the addition of ascorbic acid (ca 100 mg.) the solution was boiled for one minute. After cooling, and dilution to 50 ml. in a graduated flask the absorbance, at  $675\text{ m}\mu$  in 1 cm. cells, was measured using a Unicam spectrophotometer, model SP 600. (Thomson, 1961).

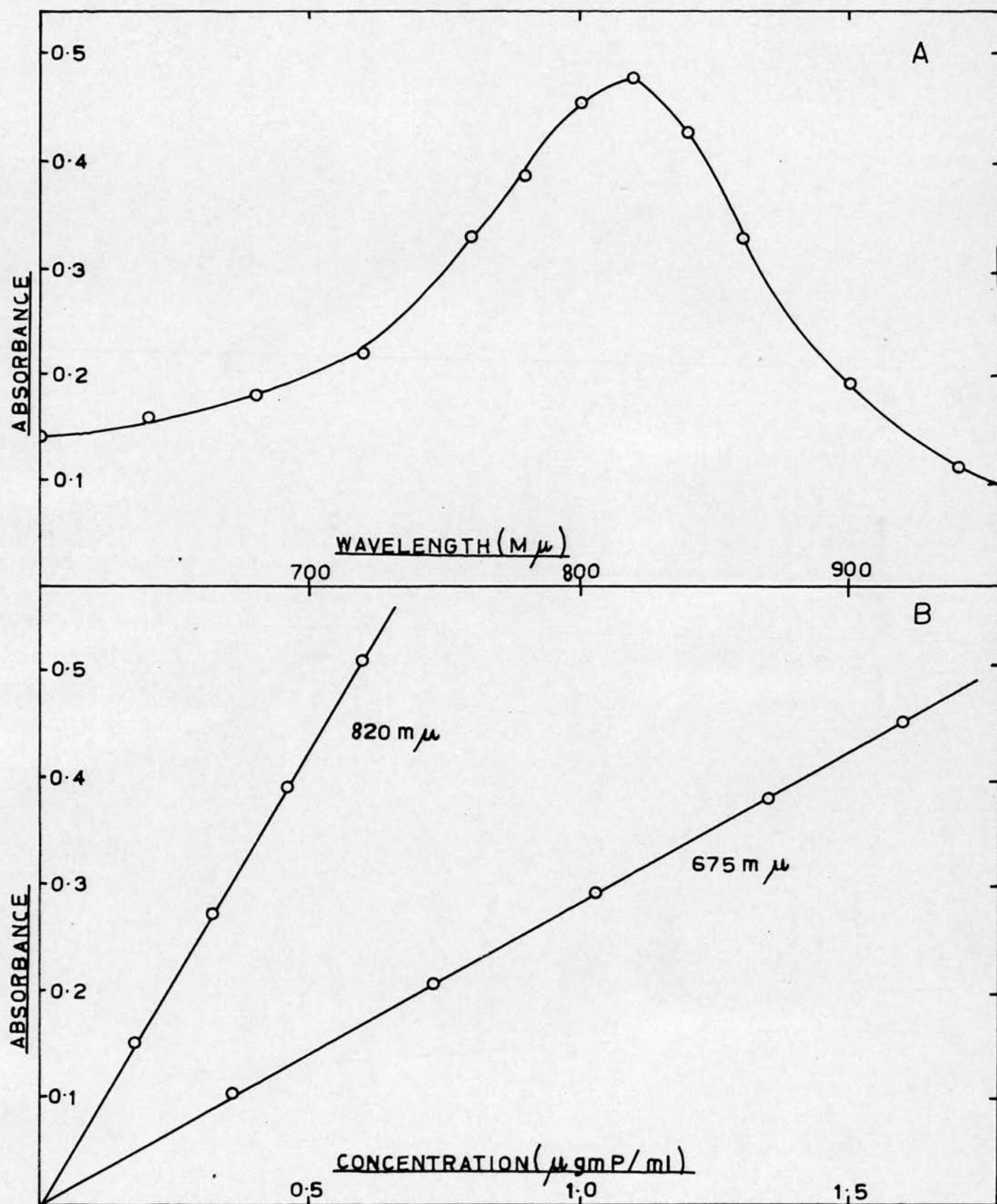
While Fogg and Wilkinson, (1958) measured the absorbance of the molybdenum blue colour using a Spekker Absorptiometer and Ilford red filter NO.608, and Thomson (1961) used a wavelength of  $675\text{ m}\mu$  the actual wavelength of maximum absorption has been quoted as  $830\text{ m}\mu$  (Bartlett, 1959) and  $820\text{ m}\mu$  (Lowry et al, 1954; Chen et al, 1956). The values were found after reduction both by ascorbic acid and 1-amino 2-naphthol 4-sulphonic acid.

A wavelength of  $675\text{ m}\mu$  was used in the earlier part of this work, but subsequent measurement of the absorption spectrum of the reduced phosphomolybdic acid showed that the wavelength of maximum absorption was in fact /

fact 820  $m\mu$  (Fig. 2.2.a) corresponding to a molar extinction coefficient of 26,500. This led to an increase, greater than twofold, in the sensitivity of the method as compared with that employed by Thomson (1961), enabled smaller quantities of polysaccharide to be used and, in particular, rendered estimation of the phosphorus content of amylose more meaningful.

Calibration graphs were obtained using aliquots of a standard solution of potassium dihydrogen phosphate (Fig. 2. 2. b).

FIG. 2.2





(d) POTENTIOMETRIC IODINE TITRATION.

A knowledge of the composition of starch and the purity of the separate components, is most conveniently obtained by estimating the amount of iodine bound by the particular polysaccharide in question.

The blue-staining amylose component binds iodine at relatively low concentrations and in amounts depending on the chain length, to form a complex in which the iodine atoms take up positions in the axis of spirally wound, amylose molecule chains. This is followed, at higher concentrations of iodine, by a secondary process of adsorption on the exterior of the amylose helices. While the presence of iodide ion is essential, the blue colouration was, under certain conditions, (low binding; low free iodine concentration) attributed by Gilbert and Marriott, (1948) to be due to the formation of a molecular ion of composition  $I_3^-$  or  $3I_2 \cdot 2I^-$ . More recent workers, (Hollo and Szejtli, 1960) have depicted the amylose-iodine complex as containing 10 -20 resonating iodine-atom chains. In considering the metallic nature of the amylose-iodine complex, Bersohn and Isenberg, (1961) observed a weak electron resonance and further, on the basis of there being about 3.9 glucose units per iodine atom, concluded that the amylose- $I_3^-$  complex contains a row of 50 - 500 iodine atoms.

Pure amylopectin, on the other hand, stains a red colour and requires a much greater free iodine concentration to reach a comparable degree of saturation. Since helix formation /

formation in amylopectin will be minimal, owing to the large number of branch points, Higginbotham's (1949) suggestion that  $I_2$  and  $I_3$  molecules may be singly adsorbed, appears to be the most likely mechanism.

The measurement of the amount of iodine bound by a starch was first put on a reliable, quantitative basis by the development of the potentiometric method by Bates, French and Rundle, (1943). Experimentally, the task was made easier by the differential refinement of Gilbert and Marriott, (1948). This latter method eliminated the necessity for a blank titration and introduced constant stirring. Further development by Anderson and Greenwood (1955) led to the apparatus used in this work.

Although the straightforward potentiometric method is widely used, it is not applicable to modified starches whose linearity has been disturbed by oxidation, dextrinisation or hydrolysis. With such materials the iodine titration curve does not have a sharp inflection point and hence the plot of bound versus free iodine does not give a linear section which can be extrapolated. Deatherage et al., (1955) have used a modified method, incorporating calcium chloride as a solvent and claim that a reliable index of relative linear character is provided. While originally designed for commercially modified starches and dextrans, the method has been satisfactorily /

satisfactorily used for the assay of linear material in a wide variety of natural starches, though incomplete solution and consequent low results were obtained with certain high-amylose starches.

In parallel with the development of the potentiometric method, various workers have employed the amperometric estimation of Larson, Gilles and Jennes, (1953) in which iodine is generated in solution from iodide ions by reaction with iodate ions. In a comparison of the methods available for determining iodine absorption, Hollo et al, (1960) claimed increased accuracy over the potentiometric titration. However, as in the earlier potentiometric system, a reagent blank is necessary.

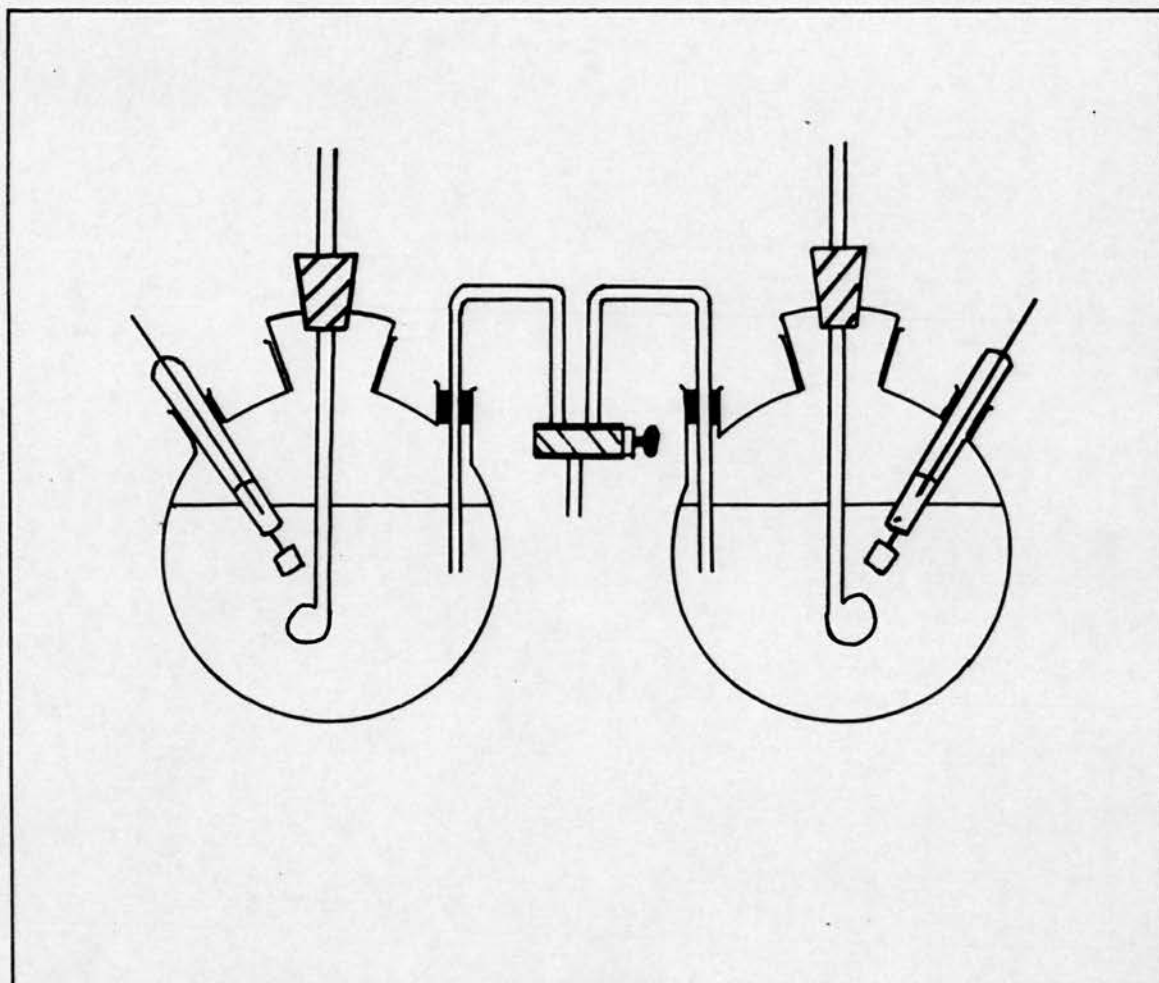
#### Apparatus.

The primary feature of the apparatus was an electrometer combining high sensitivity and high zero stability, the latter characteristic being necessary since the equilibrium free iodine concentration was measured by a null deflection method.

A polysaccharide-iodide solution and a blank-iodide solution were contained in two opposing half-cells connected by a salt bridge. The half-cells consisted of 1 litre flasks having four necks, three of which held the stirrers, the electrodes and the salt bridge, while iodine solution was added by way of the fourth neck (Fig. 2.3.). Stirring was /



FIG. 2.3



Cell Arrangement

Iodine Titration Apparatus

was continuous and the electrodes, made of platinum foil, were chosen so that no potential difference existed between them when placed in the same solution.

Despite the undesirable feature of decreasing sensitivity with increasing free iodine concentration, results were obtained reproducible to  $\pm 2\%$ .

The sample, (free from proteins, fatty acids and complexing agents) was dried in vacuo at  $60^{\circ}$  for several hours. The amount used varied with the character of the polysaccharide (amylose 3 - 5mg; starch 10 - 15mg; amylopectin 25 - 30mg.). After dissolution in sodium hydroxide (0.2N; 10ml.), the solution was brought to pH 5.85 by the addition of a previously determined volume of phosphoric acid (0.4N). A blank solution was prepared containing the same volumes of alkali and acid but no polysaccharide.

#### Titration Conditions and Procedure.

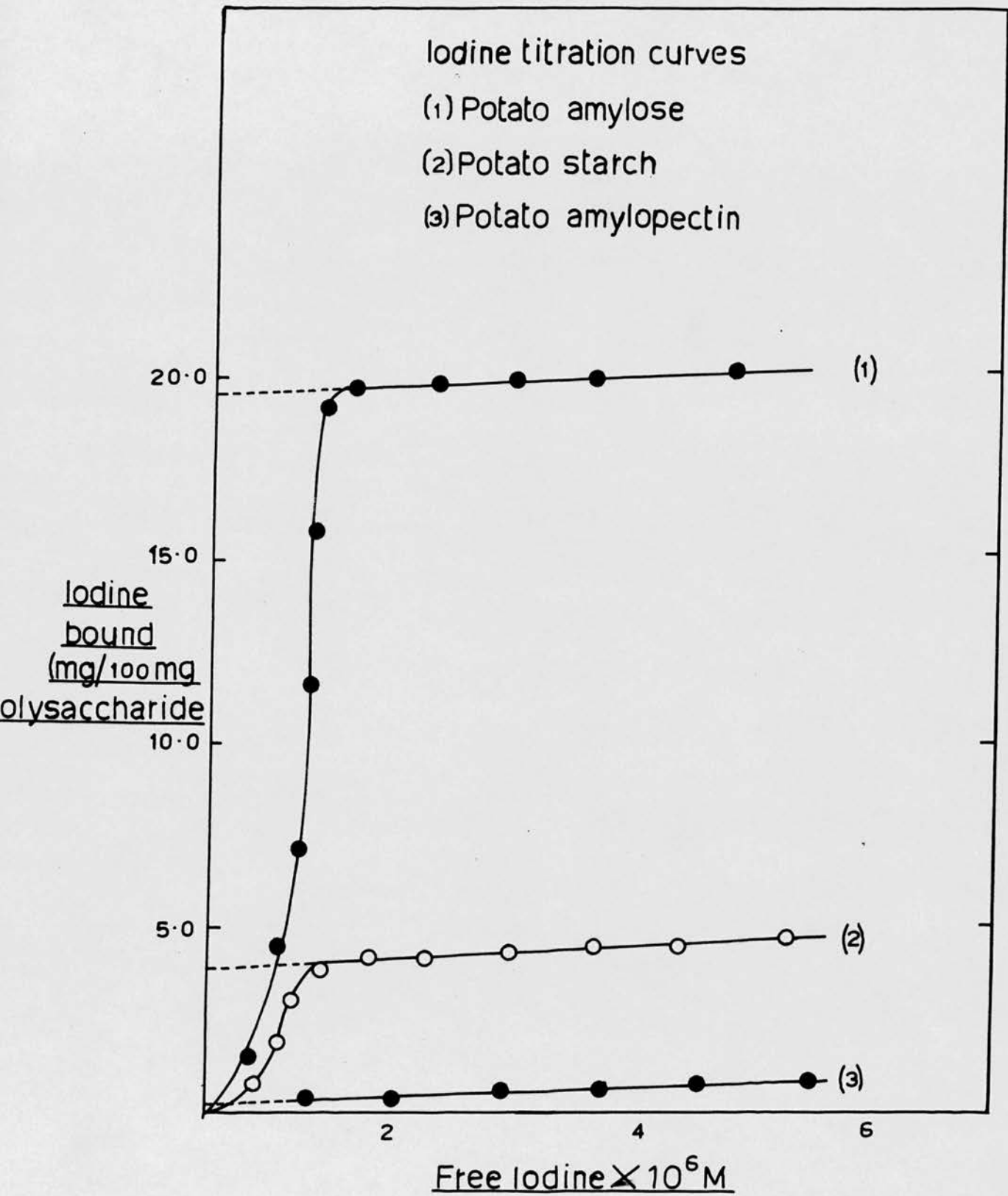
The titration conditions were:- iodide, 0.01M; pH, 5.85 and temperature,  $20^{\circ}\text{C}$ . The electrolyte solution consisted of potassium iodide (0.1M; 210ml.) and phosphate buffer ( $\frac{M}{E}$ , 30ml. pH 5.85) made up to 2 litres with distilled water. The sample solution and blank were washed into their respective cells using 25ml. of electrolyte, following the addition of 800ml. of electrolyte to each half-cell; the final volume was thus ca 850ml. The half-cells were then placed in a thermostatically controlled water bath and allowed to reach temperature equilibrium. Before /

Before commencing the titration, the circuit was checked to ensure that the preliminary off-balance potential was negligible.

Using an "Agla" syringe, small increments of iodine-potassium iodide solution (0.01M) were added to the half-cell containing the polysaccharide. Allowing a few minutes (2 - 3) for equilibrium to be reached, the potential produced by the free iodine, as indicated by the deflection on the galvanometer, was balanced by the gradual addition of iodine-potassium iodide solution to the control half-cell, until the original zero deflection position was restored. The amount of iodine bound by the sample was simply the difference between the amounts added to the sample half-cell and that added to the control half-cell. The titration curve of which typical examples may be seen in Fig. 2. 4, was obtained by plotting the iodine bound (mg./100mg. of polysaccharide) against the free iodine concentration. The "iodine affinity" of the polysaccharide was derived by extrapolation of the linear portion of the curve to zero free iodine concentration.

On this basis, a pure potato amylose binds 19.5% of its own weight of iodine. Using this figure as a standard, the "apparent amylose content" of a whole starch and the percentage amylose as an impurity in amylopectin, may be calculated.



FIG.2.4

(e) SEDIMENTATION.

When solid particles are suspended in a liquid medium they will tend to sediment under the action of the force of gravity. With materials of colloidal dimensions, however, force fields many times greater than gravity are required in order to overcome diffusion and promote a measureable sedimentation rate which, at the same time, is proportional to the molecular size. The easiest way to achieve high force fields is centrifugation at high speeds, i.e. ultracentrifugation.

The era of the ultracentrifuge began as early as 1923, when Svedberg and his collaborators first began exploiting centrifugal fields for the study of colloidal particles and macromolecules. For many years progress was slow and even in 1947, there were less than twenty ultracentrifuges in existence. However, the past 10 - 15 years have seen dramatic changes. The ultracentrifuge has become available as a reliable, commercial instrument and in consequence has become widely used as an analytical tool in several of the scientific disciplines.

Radical advances of a theoretical nature have also been made, with the result that molecular weights can now be determined with accuracy in a single experiment of short duration. The earlier classic work of Svedberg and Pedersen (1940) has been followed by several reviews of which the most comprehensive is the monograph by Schachman (1959).

The /

The ultracentrifuge provides the most convenient method of establishing whether a material contains more than one molecular weight range. Such a condition would give rise to two or more peaks on sedimentation. Primarily, however, ultracentrifugation enables the molecular weight of the solute to be determined.

The determination of the molecular weight of a solute may be considered in two broad classifications; (a) sedimentation velocity measurements in which the rate of movement of the solute is related to the molecular weight (b) sedimentation equilibrium, in which the applied centrifugal force is exactly balanced by the tendency of the molecules to diffuse. Attention in this work is confined to the former method.

#### Theory of Sedimentation Velocity.

Under the influence of a centrifugal force, the rate of movement of the particles in solution depends on particle size, shape and density, the density and viscosity of the medium and the magnitude of the gravitational field, provided no convectional or electrical forces interfere. Svedberg showed that the rate of movement of the molecules can be measured provided certain conditions are fulfilled. One essential requirement is that sedimentation should be carried out in a sectorial shaped cell spun in a radially directed centrifugal field. The two flat walls of the cell, if extended, would intersect the axis of rotation; the other two walls are perpendicular to the axis. This ensures /



ensures that molecules originally close to the wall will sediment parallel to these walls and disturbances, caused by collision with the cell side, are avoided.

After initial acceleration, the rate of sedimentation will be constant, the centrifugal force being balanced by a frictional force. At a distance  $x$  from the axis of rotation, the centrifugal force is  $w^2 x(1 - \bar{v}_e)m$  where  $w$  is the angular velocity,  $m$  is the mass of the sedimenting molecules and  $\bar{v}$  is the partial specific volume. The frictional force is the product of the velocity,  $\left(\frac{dx}{dt}\right)$  and a frictional coefficient ( $f$ ).

$$\text{Thus } w^2 x(1 - \bar{v}_e)m = f \frac{dx}{dt}$$

This may be rewritten as

$$\begin{aligned} \frac{dx}{dt} \cdot \frac{1}{w^2 x} &= \frac{m(1 - \bar{v}_e)}{f} \\ &= s. \end{aligned}$$

The sedimentation coefficient is thus defined as the velocity of sedimentation per unit force field. However, the frictional coefficient may be related to the diffusion constant ( $D$ ); at infinite dilution  $f_0 = \frac{kT}{D_0}$  and thus substitution in the above equation and multiplication by  $N$  (Avogadro's Number) gives

$$M = \frac{RTs_0}{(1 - \bar{v}_e)D_0}$$

As the above derivation makes no assumption of molecular shape, it should be universally applicable. While the partial specific volume may be easily obtained using a pyknometer /

pyknometer, the diffusion constant can be measured only by specialised techniques. For this reason it is often more convenient to eliminate  $D_0$  from the above equation; and make use of the method of Scheraga and Mandelkern (1953). By combination of viscosity and sedimentation measurements, it can be shown that

$$\beta = \frac{N S_0 [\eta] \eta_0}{M^{\frac{2}{3}} (1 - v_c)}$$

where  $\beta$  is a constant related to the axial ratio of the molecule.

#### Apparatus and Procedure.

The instrument used was a Spinco Model E, electrically driven ultracentrifuge, capable of speeds up to 60,000 r.p.m. The cell containing the solution to be examined consisted of a centrepiece with a sector shaped cavity clamped between quartz discs and held in a cylindrical housing. The cell was radially aligned in the rotor by means of scribe marks; this precaution was necessary to prevent convectional disturbances. Prior to acceleration, the steel rotor chamber was evacuated (pressure less than  $\mu$  mercury) by means of an oil diffusion pump backed by a rotary vacuum pump. High vacuum was necessary to eliminate, or at any rate minimise, thermal disturbances. During the run the temperature was maintained at  $20 \pm 0.02^\circ\text{C}$  by means of a Rotor Temperature Indicator and control unit. The Schlieren /

Schlieren optical system gave a direct measure of the variation in refractive index gradient throughout the cell. Photographs of the sedimentation boundaries were taken at known time intervals during the run.

#### Calculation of the Sedimentation Coefficient.

The photographs of the sedimentation boundaries were measured by means of a two-way travelling microscope. The distance of the maximum ordinate of the peak from a reference line was measured to the nearest 0.01 mm. After correction for a lens magnification factor, this distance was then related to the absolute distance (x) of the sedimenting boundary from the centre of rotation. The sedimentation coefficient was then evaluated from a plot of log (x) versus time, giving

$$s = \frac{2.303}{w^2} \cdot \frac{d \log x}{dt}$$

#### Dependence of the Sedimentation coefficient on Concentration.

In sedimentation, concentration dependence is connected with molecular entanglement, solvation and the hydrodynamic interactions to which the molecules are subjected in solution. The degree of dependence, however, varies considerably according to the physical properties of the macromolecules. Thus rigid, spherical molecules such as glycogen /



glycogen show very little dependence on concentration, whereas threadlike molecules such as amylose are markedly concentration dependent. Since present theories for the interpretation of sedimentation velocity experiments require the value of the sedimentation coefficient at infinite dilution, sedimentation coefficients are usually measured over a range of concentrations and the values extrapolated to zero concentration. This can best be achieved by plotting  $\frac{1}{s}$  against  $C$  which gives a straight line.

(f) VISCOSITY.

One of the most characteristic properties of a polymer is its ability to increase the viscosity of the medium in which it is dissolved. However, prior calibration is necessary before it is possible to relate the absolute molecular weight of a polymer to its viscosity. Thus, the method is primarily used as a means of comparing the changes in molecular weight which may occur in a given series of samples. That is to say, it is used as a relative measure.

The basic terms used in practical viscometry are as follows.

Relative viscosity:-  $\eta_r$  = ratio of the viscosity of the solution,  $\eta$ , to the viscosity of the solvent

$$\eta_r = \frac{\eta}{\eta_0}$$

Specific viscosity:-  $\eta_{sp}$  = relative increase of viscosity

$$= \frac{\eta - \eta_0}{\eta_0} = \eta_r - 1$$

Viscosity number:-  $\eta_c$  = specific viscosity divided by the concentration

$$= \frac{\eta_{sp}}{c}$$

The viscosity number is dependent on the concentration, hence, in order to define a quantity characteristic of a given polymer - solvent system, it is necessary to obtain the value of the viscosity number at infinite dilution.

This /

This value is known as the intrinsic viscosity or the limiting viscosity number and is denoted by  $[\eta]$ .

$$[\eta] = \lim_{c \rightarrow 0} \frac{\eta_{sp}}{c}$$

The intrinsic viscosity has the dimension of the reciprocal of concentration, which in this thesis is expressed in g./ml. as recommended by I.U.P.A.C. (1952).

While a number of empirical relations have been suggested to explain the concentration dependence of the viscosity number, the most widely used is that due to Huggins (1942)

$\eta_{sp} = [\eta] c + k [\eta]^2 c^2$  where the coefficient  $k$  is believed to be some function of solute - solvent interaction.

Although this relation cannot be applied to all polymer-solvent systems, it is generally accepted to be the most appropriate at low concentrations. It is pertinent to note that empirical equations of this type are useful only in certain limited ranges of concentration and, therefore, care should be taken in applying them to experimental results.

#### Viscosity and Molecular Properties.

The viscosity method is the simplest method used in molecular weight determinations involving dilute polymer solutions. The problem of the correlation of viscosity with /



with molecular dimensions has been approached in two ways (See Banks and Greenwood, 1963).

The first of these attempts to treat the solute molecules as rigid impermeable particles which may be approximated by spheres or ellipsoids. This method calculates the external dimensions of the solute particles from the viscosity without regard to the molecular configuration.

By visualising linear molecules as stiff rods, Staudinger, (1932) was able to relate the limiting viscosity number to the molecular weight<sup>\*</sup> by means of the function

$$\frac{\eta_{sp}}{c} = K \cdot M$$

where /

---

\* For polymolecular materials various molecular weight averages exist. The most important of those are the number average ( $\bar{M}_n$ ) and the weight average molecular weight ( $\bar{M}_w$ ).

For any system in which  $n_i$  is the number of molecules of species  $i$  and  $M_i$  is the molecular weight of the same species

$$\bar{M}_n = \frac{\sum M_i n_i}{\sum n_i} \qquad \bar{M}_w = \frac{\sum M_i^2 n_i}{\sum M_i n_i}$$

Viscosity measurements usually give an average molecular weight called the viscosity average molecular weight and defined by Flory (1943) as

$$\bar{M}_v = \left[ \frac{\sum M_i^{1+\alpha} n_i}{\sum M_i n_i} \right]^{\frac{1}{\alpha}}$$

where  $\alpha$  represents the exponent in the modified Staudinger equation. If  $\alpha = 1$  then  $\bar{M}_v = \bar{M}_w$

where  $k$  is a constant and  $M$  the molecular weight.

This equation was originally used for polymers of relatively low molecular weight. A more general equation relating the viscosity-molecular weight relationship for a large number of polymers in the molecular weight range  $10^4 - 10^6$  is

$$[\eta] = KM^\alpha$$

where  $\alpha$  is a constant and a function of the geometry of the molecule in solution and has values between 0.5 and 2.0 for tightly curled and rigidly extended molecules respectively, (McGoury and Mark, 1938).  $K$  and  $\alpha$  can both be found by fractionating a polymer into samples of different molecular weights and determining the weight average molecular weight ( $\bar{M}_w$ ) for each sample. A graph of  $\log [\eta]$  versus  $\log(\bar{M}_w)$  then gives a straight line of slope  $\alpha$  and intercept  $K$  on the  $\log [\eta]$  axis. These constants must be determined for each polymer-solvent system.

The viscosity of real polymer molecules is, however, most often interpreted on the basis of the random-coiled chain model which has been developed from the work of Kirkwood and Riseman, (1948) by Flory and Fox, 1953). By consideration of the distribution of chain elements and their interactions Flory and Fox showed that

$$[\eta] = KM^{\frac{1}{2}}\alpha^3 \quad \text{where } K = \phi \left( \frac{\bar{r}_0^2}{M} \right)^{\frac{3}{2}}$$

$\phi$  is a universal constant, independent of both polymer and solvent, with a value of  $2.1 \times 10^{21}$  and  $(\bar{r}_0^2)^{\frac{1}{2}}$  is the unperturbed /

unperturbed value of the root mean square end to end length of the chain. The factor  $(\frac{r_0^2}{M})$  is independent of molecular weight for a given polymer but  $\alpha$ , known as the volume expansion factor, is dependent on molecular weight.

It must be emphasised that dependence of  $[\eta]$  upon molecular weight can only be expected for linear molecules. For compact, highly branched molecules,  $[\eta]$  may well be constant over a wide range of molecular weight.

### Experimental

The viscometers used in this work were of the modified Ubbelohde type (Ubbelohde, 1937; David and Elliott, 1949).

In a capillary viscometer of this type, the viscosity is given by

$$\eta = Kdt - \frac{Bd}{t}$$

where  $d$  is the density of the liquid,  $t$  is the time of flow, and  $K$  and  $B$  are the viscometer constant and kinetic-energy correction factor, respectively. The kinetic-energy factor is necessary to correct for the finite velocity of the liquid leaving the capillary. This correction is made negligible by having a long flow time ( $t > 200$  sec.). Therefore for the solution

$$\eta = Kdt \text{ and for the solvent}$$

$$\eta_0 = Kd_0t_0$$

The relative viscosity is therefore given by

$$\eta_r = \frac{Kdt}{Kd_0t_0} \text{ and the specific viscosity}$$

by /



by

$$\eta_{sp} = \frac{dt - d_0 t_0}{d_0 t_0} \quad \text{For a dilute polymer solution } d = d_0$$

$$\text{therefore } \eta_{sp} = \frac{t - t_0}{t_0}$$

Having thus obtained by experiment the values of  $\eta_{sp}$  for several concentrations, a graph of  $\frac{\eta_{sp}}{c}$  against  $c$  will give a plot from which  $[\eta] = \lim_{c \rightarrow 0} \frac{\eta_{sp}}{c}$  can be determined.

### Procedure

All solutions were filtered through G4 sintered glass filters before use. The viscometer (Fig. 2. 5) was clamped in a vertical position on a brass stand and determinations were carried out in a constant temperature bath at  $25 \pm 0.01^\circ\text{C}$ .

Two alternative procedures were employed.

(a) A volume of solvent ( $> 10\text{ml.}$ ) was placed in the viscometer and the flow time between two marks measured. Increments of polymer solution were added and the flow times found.

(b) A volume of polymer solution ( $> 10\text{ml.}$ ) was placed in the viscometer, the flow time determined; the flow times for subsequent additions of solvent were then found.

Although method (a) provided a check on solvent flow time and gave better spacing of concentrations, method (b) was preferred in most cases, because a much smaller volume of polysaccharide solution was required.

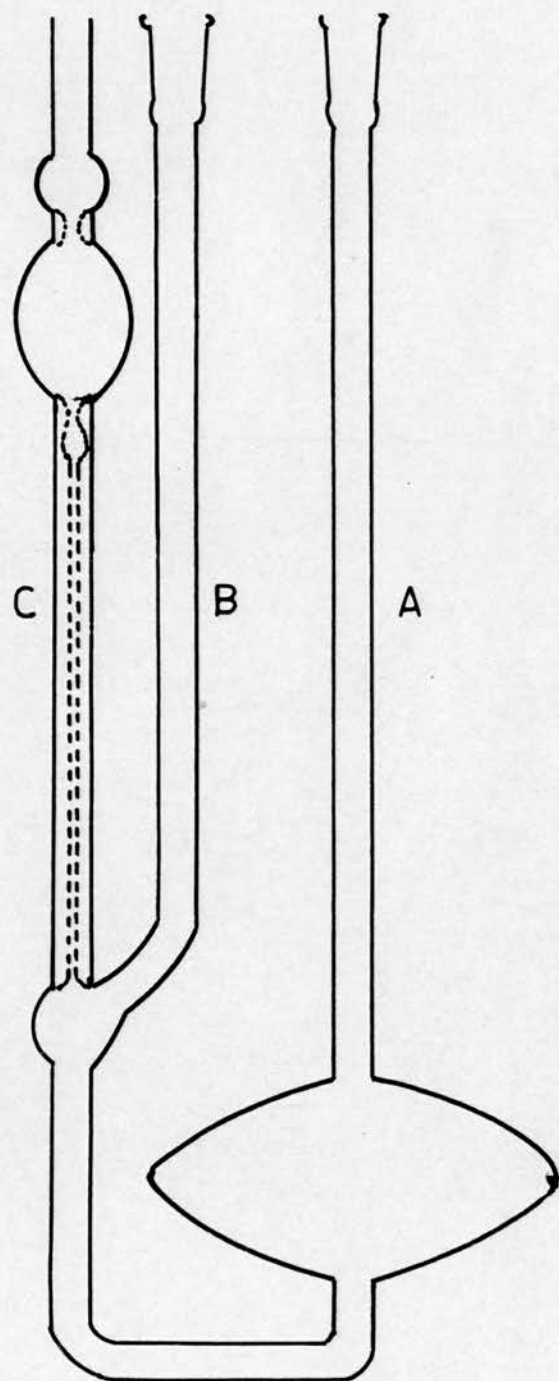
Solvent and solution were added by pipette down the tube /

tube A (Fig. 2.5). The pipette used had a long stem so that the liquid could be placed in the bulb without any contact with the sides of the tube. Closing tube B with a glass stopper and applying pressure to tube A using nitrogen from a cylinder, forced the liquid up the capillary tube. After removal of the stopper in tube B, the flow time was measured by stop-watch to the nearest 0.01 sec. Efficient mixing of solvent and solution was achieved by directing a gentle stream of nitrogen down tube B.

The concentration of the polysaccharide solution was determined by hydrolysis of portions of the solution to glucose and estimation of the glucose by the alkaline ferricyanide - ceric sulphate method.

FIG. 2.5

The Modified Ubbelohde Viscometer





S E C T I O N   3

STUDIES ON THE FRACTIONATION OF POTATO STARCH.

### INTRODUCTION.

In demonstrating that amylose forms insoluble complex compounds with the higher alcohols, Schoch (1942) set the trend for much of the research on starch fractionation carried out in the subsequent two decades. The field of starch fractionation has been covered in reviews by Schoch (1945), Greenwood (1956) and Muetgeert (1961).

Basically, starch fractionation falls into two broad classifications, (1) methods involving intact starch granules e.g. aqueous or alkaline leaching and (2) methods involving complete dispersion of the granules. Although separation of the components from a dispersion may be variously achieved by selective retrogradation, electrophoresis or chromatography, by far the most effective method entails selective precipitation of the amylose component by a polar organic substance. Whereas it is relatively simple to obtain pure amylose, the removal of residual amylose from the amylopectin fraction is much more difficult. It is therefore essential that the first precipitation should give amylopectin of maximal purity.

Following the work of Schoch (1942), the most widely used complexing agents are alcohols. Whistler and Hilbert (1945), however, have suggested that any water-soluble compound possessing either donor or acceptor groups capable of hydrogen bond formation is a suitable complexing agent. These authors have reported the use of nitrobenzene and nitroparaffins. Thymol and cyclohexanol were found by Bourne et al (1948) to be suitable complexing agents for /

for the amylose of potato starch. Greenwood (1956) reported that fractionation with thymol followed by butanol yields both amylose and amylopectin fractions of greater than 99% purity.

Various workers have also examined the comparative efficiency of fractionating agents. For instance, Hopkins and Jelinek (1948) have studied the relative precipitating efficiency of butanol, thymol and thymol followed by cyclohexanol for potato amylose. There was scarcely any difference between the products obtained. Higginbotham and Morrison (1948) studied the use of butanol, pyridine and isopentyl alcohol and concluded that pyridine and butanol were comparable, but isopentyl alcohol was not so efficient. Although the relative efficiency of many complexing agents is known in general terms, quantitative information is sparse. In Section 3, II, therefore, an attempt is made to compare the relative efficiency of some of these conventional complexing agents.

In all the above work precipitating agents have been used at their respective saturation values of concentration in aqueous solution and until the work of Muetgeert (1961) virtually no attempt had been made to investigate the influence of the concentration of the complexing agent upon the course of complex formation. Perhaps the only exception to this generalisation is the work of Bourne et al (1948); the results of these authors, however, did not indicate any considerable influence of concentration upon the formation of the amylose complex, at least in the case of cyclohexanol. The researches of Muetgeert (1961), on the other hand, demonstrated /

demonstrated that each complexing agent possesses its own specific region of concentration, termed the "critical concentration", in which it shows optimum fractionating properties. At these concentrations, the rate of precipitate formation and the iodine affinity of the precipitate are at a maximum. The critical concentration is dependent not only on the amount of amylopectin present but also on the chain-length of the amylose. It is to be noted that Muetgeert (1961) restricted his investigations to potato starch.

Muetgeert (1961) also drew attention to the possibility of fractionating starch using chloral-hydrate and evolved a procedure which gave amylose products of low iodine-affinity and relatively insoluble amylopectin fractions. This method of fractionation is further examined in Section 3, I.

The concept, expressed by Bear (1944), that a polar or polarisable molecule is necessary for the formation of insoluble complexes with amylose, was generally accepted until fairly recently. However, French et al (1963) made a survey of the ability of various hydrophobic substances to act as starch fractionating agents. The procedure used was essentially the same as that for fractionation with aliphatic alcohols. Initially, effectiveness was judged by the temperature at which amylose complex formation was first noted during the cooling of a starch solution containing the reagent. Subsequent purity of the fractions was estimated from determinations of the iodine-affinity and the  $\beta$ -amylolysis limit. Both the amylose and amylopectin fractions were found to be comparable to /



to the products obtained when using n-amyl alcohol as the fractionating agent. The most effective hydrophobic complexing agent used by French et al was found to be 1,1,2,2-tetrachloroethane. Other efficient precipitants were cyclohexanol, carbon tetrachloride, chloroform and fluorobenzene.

It is of interest to note that the initial separation of the amylose complexes was achieved by centrifugation at 2000-3000 r.p.m. Centrifugation at much higher speeds is normally desirable to obtain efficient separation of, for example, an amylose-thymol complex.

French et al, applying the Muetgeert concept of critical concentration, also tested the possible merit of using a deficiency of the hydrophobic complexing agent. For 1,2-dichloroethane, the amylose product had a lower iodine-affinity than that obtained with the saturation level of precipitant. This was attributed to poor separation from the amylopectin fraction. On the other hand, an amylose of superior iodine-binding power was obtained when using a deficiency of 1,1,2,2-tetrachloroethane.

The mechanism of complex formation was attributed, on the bases of X-ray diffraction experiments, to be due to the formation of helical inclusion compounds.

Some aspects of the fractionation of starch by hydrophobic complexing agents are examined in Section 3, III.

SECTION 3, I.

CHLORAL HYDRATE FRACTIONATION.

EXPERIMENTAL.

Materials.

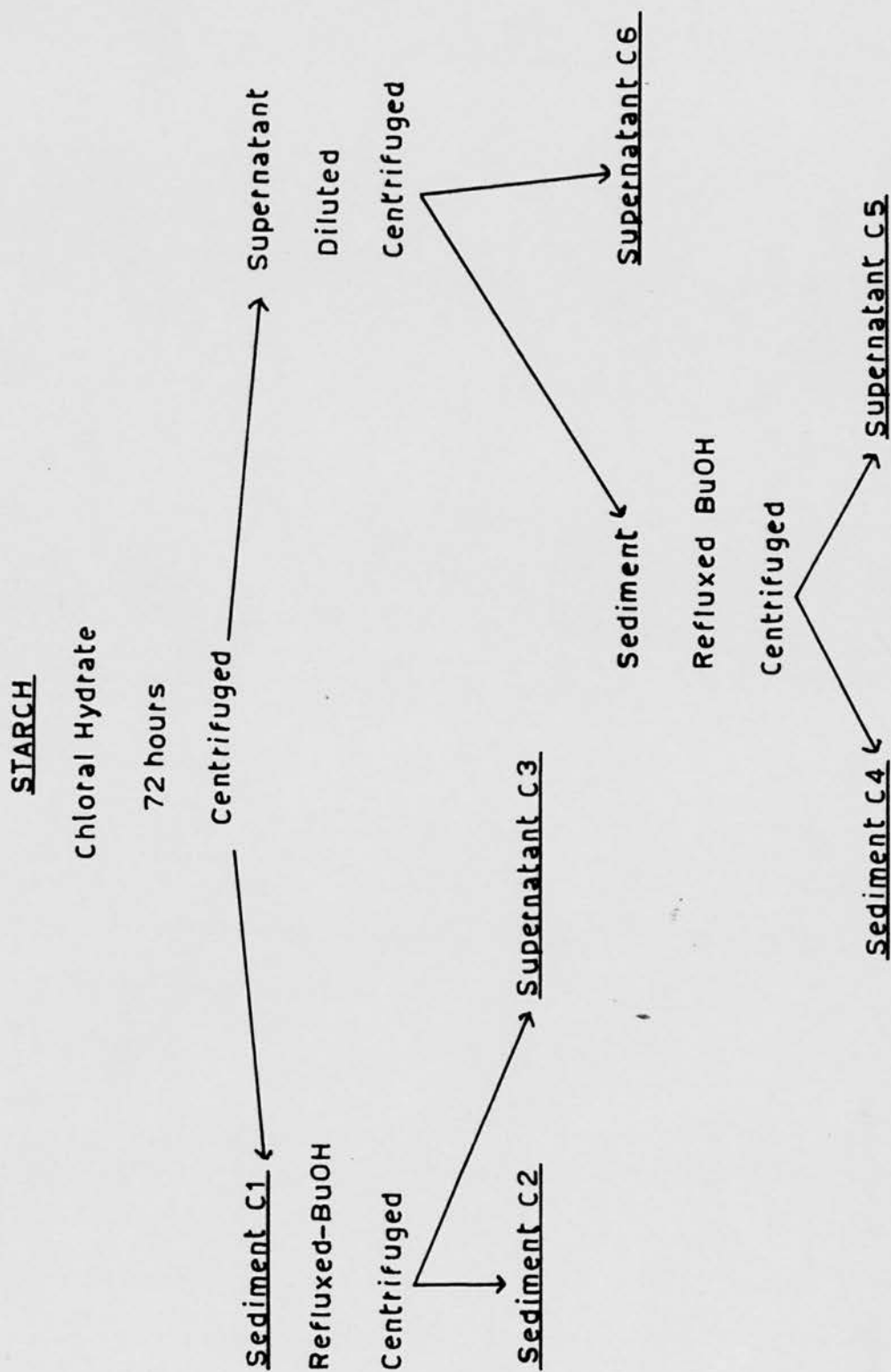
The starch used in the following fractionation experiments was obtained from the Pentland Crown variety of the potato tuber. The 'total' starch was fractionated into granules of different sizes by the method of Decker and Hoeller (1962). The granules used (Sedimentation time 2 minutes; see Section 6, II) had a number-average diameter of  $45\mu$ ; iodine-binding determinations showed the amylose content of the granules to be 21.5%. Prior to fractionation the granules were exhaustively defatted by the method detailed in Section 2.

Fractionation of the Granules.

The method used was essentially that of Muetgeert (1961).

Chloral hydrate (120 g.; 100%) was dissolved in distilled water (60 ml.) and buffered by the addition of sodium acetate dihydrate (4 g.). A suspension of starch (10 g.) in distilled water (20 ml.) was slowly added at room temperature, with constant stirring. The suspension was kept in the dark at 20°C for 72 hours. The resultant solution was centrifuged (10,000 x g.) and the gelatinous precipitate washed several times, first with chloral hydrate (30%), then with distilled water. The polysaccharide (Fraction Cl; see Fig. 3, 1) was redissolved in boiling, oxygen-free water and recovered by /

FIG.3.1



by freeze-drying the resultant solution. A portion of the solution obtained prior to freeze-drying was refluxed with n-butanol, yielding a precipitate (C2) and a further subfraction (C3) which was recovered from the supernatant solution by freeze-drying.

The chloral hydrate washings of fraction C1 were combined with the supernatant from the first centrifugation; the solution was diluted with water till the chloral hydrate content was 5-8% and kept for 24 hours. The precipitated amylose was separated in an ordinary laboratory centrifuge (1500 x g.). After being washed several times with water, the amylose was redissolved in boiling water (nitrogen atmosphere) and precipitated with n-butanol, yielding fraction C4. The supernatant solution was freeze dried to yield fraction C5.

The major amylopectin fraction was precipitated by the addition of several volumes of ethanol to the mother liquor remaining after centrifugation of the amylose. The precipitate was washed several times with distilled water to remove excess chloral hydrate and then redissolved in boiling water (nitrogen atmosphere). Freeze-drying of the solution yielded fraction C6.

#### Characterisation of the Fractions.

Characterisation of the fractions with respect to iodine-affinity, limiting viscosity number,  $\beta$ -amylolysis limit and  $\beta + Z$  amylolysis limit was carried out as detailed in Section 2.





# RESULTS AND DISCUSSION.

The properties of the fractions are shown in Table 3. 1.

TABLE 3. 1.

Chloral Hydrate Fractionation Products.

Fraction	Iodine Affinity $\phi$	Amylose (%) $\epsilon$	$[\eta]$ $\dagger$	$\beta^1$ $\%$	$\beta^2$ $\%$
C1	2.80	14	-	68	-
C2	15.0	77	250	71	89
C3	0.02	0.1	-	50	-
C4	17.2	88	440	78	95
C5	0.09	0.5	-	55	-
C6	0.08	0.4	-	53	-

$\phi$  Expressed as mg.  $I_2$  bound/100 mg. of polysaccharide.

$\epsilon$  Calculated from iodine affinity.

$\dagger$  Determined in 0.15 M potassium hydroxide at 25°C.

$\%$  Percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase (2)  $\beta$ -amylase + Z-enzyme.

The first fraction obtained from the chloral hydrate fractionation (C1) consists largely of amylopectin contaminated with about 14% of amylose. Removal of the amylose by precipitation with n-butanol gave an amylopectin (C3) binding very little iodine and thus of high purity. The  $\beta$ -amylolysis limit (50%) further indicates the complete absence /

absence of linear amylose-type material. The precipitated amylose subfraction (C2), however, is contaminated by amylopectin. This is indicated by the iodine-affinity (15.0 mg. I<sub>2</sub> bound/100 mg. polysaccharide), the comparatively low intrinsic viscosity and the incomplete conversion into maltose under the combined action of  $\beta$ -amylase and Z-enzyme.

The major amylose fraction (C4) is relatively pure. The iodine affinity indicates an amylose content of 88%. This result is higher than values quoted by Muetgeert (1961). In Muetgeert's work, however, the rather low iodine-affinity could be due, not only to the presence of amylopectin but also to the inhibiting effect of chloral hydrate on the formation of the amylose-iodine complex (Schaer, 1896). The portion of fraction C4 used to determine the iodine-affinity was dissolved in water, precipitated with ethanol and washed several times with water. The precipitated amylose was then redissolved in hot water and reprecipitated with n-butanol. This procedure was repeated to ensure complete removal of chloral hydrate. The success of the purification process was indicated by an increase in the iodine-affinity from an initial value of 13.8 to a final value of 17.2. The former value is within the range (13.2 - 14.3) quoted by Muetgeert (1961); the low-iodine affinities quoted by the latter author would, therefore, appear to be primarily due to interference by the chloral hydrate. Similar precautions to remove chloral hydrate were also taken before determining the iodine-affinity of the other amylose and amylopectin fractions. In agreement with Muetgeert, the intrinsic viscosity of /

of the major amylose fraction is high. Taken in conjunction with the  $\beta$  + Z-amylolysis limit of 95%, the relative purity of the amylose, and hence the validity of the iodine-affinity quoted in Table 3. 1, is beyond question.

In direct contrast to Muetgeert's observations, recrystallisation serves to increase, not decrease, the viscosity of the amylose samples. For instance, a second recrystallisation of fraction C4 yielded a product having an iodine-affinity of 18.1 (93% amylose), an intrinsic viscosity of 480 and  $\beta$ - and  $\beta$  + Z-amylolysis limits of 81 and 97% respectively. In view of this, it is suggested that the recrystallisation conditions (by dissolution in M potassium hydroxide at 20°C) used by Muetgeert caused degradation of the amylose. The above amylose obtained by chloral hydrate fractionation compares favourably with the amylose obtained from the same sample of starch by fractionation with thymol followed by recrystallisation with butanol. ( $\beta$ -amylolysis limit - 82%; + Z-amylolysis limit 90%; limiting viscosity number 520; see Section 3, II). The viscosity of the chloral hydrate product (480) is lower than that of the thymol-butanol product (520) and while the former value is comparable to those obtained with other precipitants (see Section 3, II) it is possible that, despite the initial use of a buffer, a limited amount of degradation of the amylose has occurred.

Fraction C5 consists of pure amylopectin; the major amylopectin fraction (C6) is also pure. The iodine-affinity indicates the presence of only 0.4% of amylose. In addition,  
a /

a solution of the amylopectin gave a red colour ( $\lambda$  max 550-560 m $\mu$ ) on staining with iodine. The average length of unit-chain, determined by periodate oxidation, was found to be 20 glucose units. While this value is somewhat lower than most literature values for potato amylopectin, the results presented in Section 6 indicate that the chain-length of potato amylopectin is dependent on, and bears an inverse relation to, the size of the granule from which the amylopectin is obtained. In this instance, a uniform fraction of fairly large granules was used.

All the amylopectin-type fractions obtained dissolved readily in hot water. This observation is in direct contrast to the comment by Muetgeert (1961) that amylopectins obtained by chloral hydrate fractionation were insoluble in hot water and even in cold N potassium hydroxide solution. However, the insolubility may be attributed to the fact that, in the latter case, the amylopectins were recovered finally by precipitation with ethanol. The samples were then washed thoroughly with ethanol. In these laboratories, it has been found (Greenwood, 1956) that precipitation and, more especially, subsequent drying with ethanol, yields relatively insoluble products, regardless of the method used for fractionation.

In general, it may be concluded that fractionation by chloral hydrate can yield both amyloses and amylopectin of high purity. However, the method should incorporate stringent precautions to eliminate chloral hydrate from the final products and therefore this method, while of intrinsic interest, is unlikely to replace existing, more convenient, methods for the fractionation of starch.



### SECTION 3. II.

#### FRACTIONATION BY POLAR PRECIPITANTS.

##### EXPERIMENTAL.

In order to obtain more precise control over the experimental conditions, the efficiency of the various polar precipitants was compared by using an amylose-thymol complex as the fractionation medium. This procedure facilitated experimental manipulations, reduced the number of variables operative and, in particular, removed the uncertainties inherent in obtaining uniform starch dispersions. For instance, more accurate control of the actual concentration of polysaccharide in solution was possible.

##### Materials.

The starch granules used for these experiments were obtained from the same sample as was used for the chloral hydrate fractionation (see Section 3. I). The granules were deproteinised and defatted as detailed in Section 2.

##### Fractionation of the Granules.

The amylose-thymol complex was obtained from a large-scale fractionation of the granules, as follows:-

Distilled water (4 litres) was boiled, for 1 hour, in a 5 litre, three-necked flask fitted with a stirrer, condenser and a gas-inlet tube through which was passed a stream of nitrogen (oxygen-free). Potato starch (20 g.) was added, in a methanolic slurry, to the boiling, oxygen-free water and heated under reflux until /

until a uniform dispersion was obtained. After allowing the dispersion to cool to 60°C, thymol (1 g./litre; in methanolic solution) was added. The dispersion was then allowed to cool slowly to room temperature. All stages of the procedure were carried out with stirring and the passage of nitrogen. The cooled dispersion was filtered through glass wool and stored for three days in a nitrogen atmosphere. The amylose-thymol complex was removed by use of a Serval, continuous-flow, high-speed centrifuge (15,000 x g.). The densely packed precipitate was then homogenised by shaking with oxygen-free water and stored.

The purity of the amylose-thymol complex was determined by estimation of the iodine affinity. The limiting viscosity number was determined in 0.15M potassium hydroxide and a further indication of the purity was obtained by estimation of the percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase and (2)  $\beta$ -amylase + Z-enzyme.

#### Fractionation of the Amylose-Thymol Complex.

In preliminary experiments, samples of the amylose-thymol complex were centrifuged for 10 minutes in a laboratory centrifuge (graduated centrifuge tubes; 1500 x g.). The 'volumes' of packed complex were noted. After dispersion in boiling water (150 ml.), aliquots (3 x 1 ml.) were withdrawn for determination of the polysaccharide concentration by the method detailed in Section 2. Several estimations were required to determine the 'volume' of complex necessary to give a standard solution of the polysaccharide (150 /

(150 ml.; 0.1% concentration). In subsequent experiments the concentration was checked and was found to be consistent to within  $\pm 4\%$ . Amylose-thymol complex (150 mg.) was dissolved in boiling water (150 ml.; oxygen-free) in a 250 ml., three-necked flask fitted with a condenser, stirrer and a gas-inlet tube. The polar complexing agent was added, the solution allowed to cool slowly, with stirring, to room temperature and the whole stored for 24 hours in a nitrogen atmosphere. The resultant precipitate was separated by centrifugation (10 minutes; 1500 x g.). The material remaining in the supernatant solution was weighed after recovery by freeze drying. The yield of precipitate was estimated by subtraction of the weight of the freeze dried product from the total weight of polysaccharide originally in solution.

#### Characterisation of the Products.

The products of fractionation were characterised with respect to their limiting viscosity number in 0.15M potassium hydroxide and the percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase and (2)  $\beta$ -amylase + Z-enzyme.

## RESULTS AND DISCUSSION.

The initial amylose-thymol complex obtained from the large-scale fractionation had the following properties:- iodine affinity - 14.2; limiting viscosity number - 350;  $\beta$ -amylolysis limit - 70% and  $\beta$  + Z-amylolysis limit 87%. The iodine-affinity indicates that the complex was only ca 72% pure (c.f. Cowie and Greenwood, 1957c).

The results obtained using various polar precipitants are shown in Table 3. 2. In experiments 1 and 2, equal amounts of thymol were added to solutions containing the original amylose-thymol complex in concentrations of 0.5 and 1.0 mg./ml. respectively. The first point of interest in these experiments is that, even when using the same precipitant as was originally used for the starch dispersion, complete reprecipitation of the polysaccharide does not occur. This suggests that the amount of polysaccharide precipitated is a function of the concentration of the starch dispersion. In experiments 1 and 2, however, different concentrations of the thymol-amylose complex lead to essentially identical products and, more especially, identical yields of the products. While a concentration effect may be operative, it must be noted that there is another essential difference between the dispersion and recrystallisation conditions. In the former, amylose is precipitated in the presence of a predominance of amylopectin, while under the recrystallisation conditions, amylose itself is the predominant component in solution.

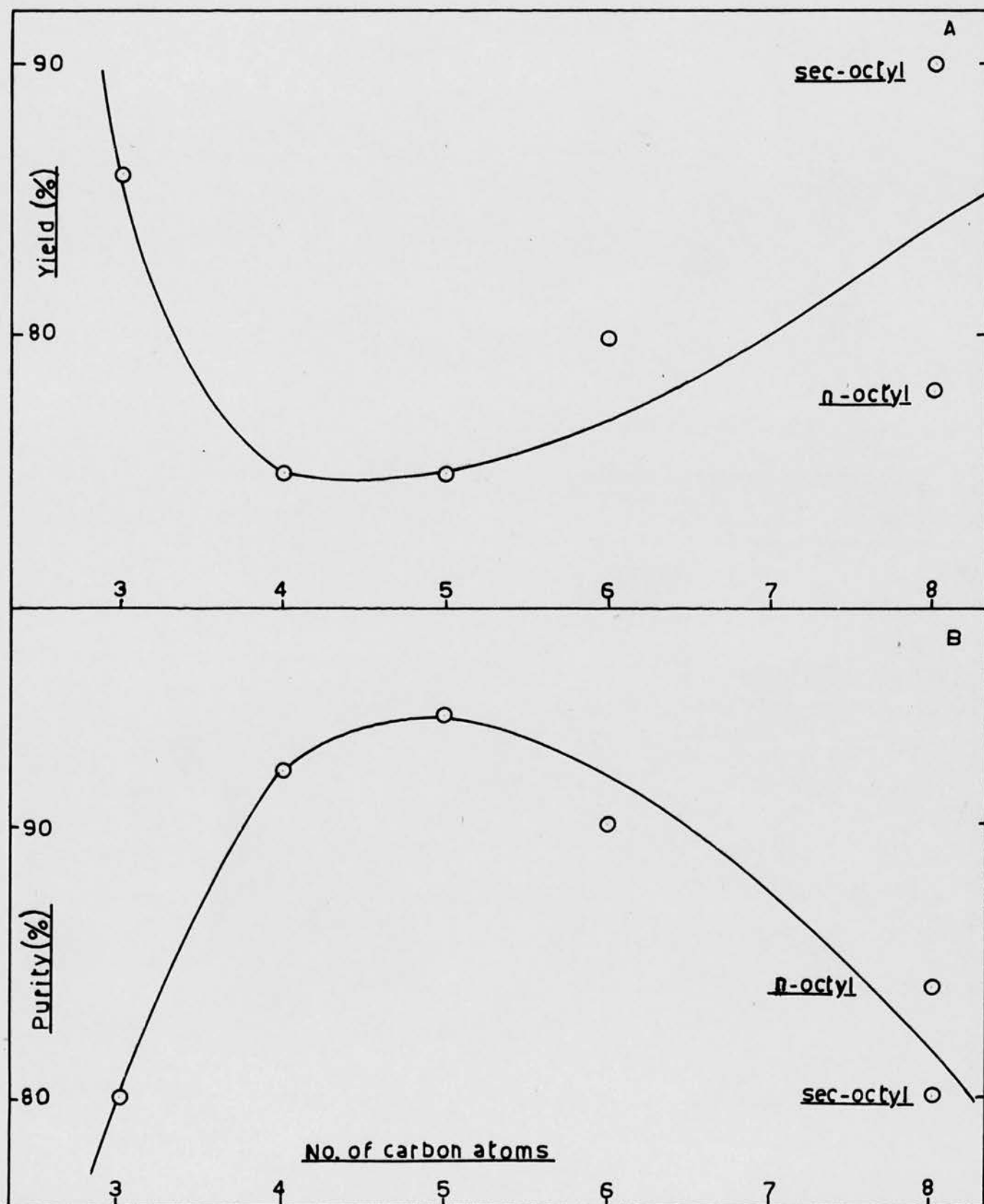
Experiments 3-11 show the effect of recrystallisation using different /



different primary alcohols from the homologous series of aliphatic alcohols. Some of the isomeric branched and secondary alcohols are also included. Considering first the linear, normal alcohols, it may be seen that the yield obtained appears to vary with the length of the carbon chain. Normal propyl alcohol precipitated ca 86% of the initial polysaccharide, while n-butyl alcohol gave a yield of 75-80%. A low yield (75%) was also obtained with n-amyl alcohol, but on further increase in the length of the carbon chain, there appeared to be a greater amount of material precipitated. These observations are better represented by the graph shown in Fig. 3. 2a, in which the yield obtained appears to be at a minimum value for chains of 4 and 5 carbon atoms. While Bear (1944) came to the conclusion that the qualitative nature of the complex (based on X-ray diffraction experiments) is not dependent on the length of the carbon chain, the above experiments indicate that n-butanol and n-amyl alcohol are more specific in their action as amylose precipitants than the other members of the series. This is more apparent when the purity of the complexes is considered. Fig. 3. 2b shows a maximum corresponding to n-amyl alcohol and, although the values are approximate ones calculated from the  $\beta$ + 2-amylolysis limits, the curve is in this case quite pronounced. It is appropriate, in this context, to point out that Schoch (1945), when placing the various precipitants then used in an arbitrary classification, preferred n-amyl alcohol.

The branched-chain members of the series were, in general, not so efficient. For instance, in experiments 3 and 4, the initial amount /

FIG.3.2.



amount of each precipitant added was 5%. No precipitation occurred. Subsequent stepwise addition of the alcohols, at 24 hour intervals, gave amylose precipitates when 15% and 30% of n-propyl alcohol and iso-propyl alcohol, respectively, had been added. Iso-propyl alcohol is thus much less efficient than n-propyl alcohol. Iso-propyl alcohol is also inefficient in the sense that coprecipitation of some amylopectin occurred; this is indicated by the  $\beta + Z$ -amylolysis limit being less than 100%. Iso-butyl alcohol (experiment 7), used at its saturation concentration, precipitated slightly more polysaccharide (82%) than did n-butanol (80%), but the difference in yields is not sufficiently large to be significant. However, the lower  $\beta + Z$ -amylolysis limit indicates the precipitation of more branched material by iso-butyl alcohol. The difference in the behaviour of the branched-chain alcohols may possibly be caused by the necessity for the amylose molecule to adopt an expanded helical form with more than six glucose residues per turn of the helix. This alternative type of conformation has been suggested by Bear (1944) and Zaslow (1963) has since confirmed that, in the case of the tert-butyl alcohol - amylose complex, there are indeed seven anhydroglucose units per turn of the helix.

Experiments 5 and 6 show the effect of recrystallisation using both the critical concentration and the saturation concentration of n-butanol. The critical concentration of precipitant was found to precipitate not only the greater quantity of amylose but also some branched material. This is indicated by the smaller  $\beta + Z$ -amylolysis limit. It would appear, from these two experiments, that the amylose-butanol complex is more soluble in water saturated with butanol than in /

in water with only the critical concentration of butanol in solution.

Chloral hydrate (experiment 12) was comparable to n-butanol both in the amount and characteristics of the product obtained. However, the rather low intrinsic viscosity may indicate that limited degradation of the amylose has occurred. Phenol (experiment 13) and pyridine (experiment 14) both precipitated large quantities of polysaccharide. However, no precipitate was formed by the latter reagent until 25% had been added. This result is somewhat at variance with the observation of Higginbotham and Morrison (1948) who concluded that n-butanol and pyridine were comparable in efficiency as starch precipitants. Quinoline (experiment 15) gave products similar to those obtained with n-octyl alcohol. Carvacrol (experiment 16), differing from thymol only in the position of the hydroxyl group, was slightly more efficient as a quantitative precipitant.

In general terms, the values for the limiting viscosity numbers of the various complexes reflect the purity of the precipitate. In particular, in experiment 6, the highest value for the limiting viscosity number is coincident with a  $\beta + Z$ -amylolysis limit indicating almost complete freedom from branched material.

Precipitates were formed readily and quickly, on cooling, with the following precipitants:- thymol, n-butanol, iso-butyl alcohol, n-amyl alcohol, n-hexyl alcohol, n-octyl alcohol, chloral hydrate, phenol, quinoline and carvacrol. The precipitate formed by chloral hydrate was more 'granular' in nature than the flocculent complexes formed by the alcohols; pyridine gave a fine precipitate more /



more akin to that usually formed on precipitation with ethanol.

Although this preliminary investigation provides some useful comparative results, much information is still required particularly with regard to the effects of the concentration of the complexing agent; the effects of varying starch dispersion concentrations also require further investigation.

TABLE 3.2.

Properties of the Complexes.

Experiment	Precipitant	Concentration (g/100 ml. at 20°C)	Yield (%) $\alpha$	$\beta_1$ $\alpha$	$\beta_2$ $\alpha$	Purity (%) $\theta$	$[\eta]$
1	thymol	0.088	85	77	93	86	440
2	thymol	0.088	85	77	91	82	445
3	n-propyl alcohol	15.0	86	72	90	80	440
4	iso-propyl alcohol	30.0	50	83	96	92	430
5	n-butyl alcohol	4.2 $\mp$	80	82	96	92	520
6	n-butyl alcohol	7.9	75	85	98	96	550
7	iso-butyl alcohol	9.5	82	76	92	84	420
8	n-amyl alcohol	2.7	75	83	97	94	490
9	n-hexyl alcohol	0.3 $\mp$	80	80	95	90	430
10	n-octyl alcohol	1.0	78	80	92	84	455
11	sec-octyl alcohol	1.0	90	74	90	80	390
12	chloral hydrate	5.8 $\mp$	82	80	97	94	450
13	phenol	2.5 $\mp$	88	75	90	80	400

14 /

TABLE 3.2. (Continued).

Experiment	Precipitant	Concentration (g/100 ml. at 20°C)	Yield (%)	$\beta$ 1	$\beta$ 2	Purity (%)	$[\eta]$
14	pyridine	25.0	88	75	91	82	385
15	quinoline	0.6 $\pi$	78	81	93	86	460
16	carvacrol	1.0	90	73	89	78	380

\* Critical concentration (see Muetgeert, 1961).

† Expressed as a percentage of the amylose-thymol complex.

†† Percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase

(2)  $\beta$ -amylase + Z-enzyme.

e Calculated from  $\beta$  + Z-amylolysis limit.

SECTION 3. III.

FRACTIONATION BY NON-POLAR PRECIPITANTS.

EXPERIMENTAL.

Materials.

The starch granules used for these experiments were obtained from the same sample as was used for the chloral hydrate fractionation (see Section 3. I). The granules were deproteinised and defatted as detailed in Section 2. Samples of some of the hydrophobic compounds were kindly supplied by Dr. D. Leaver.

Fractionation of the Granules.

Starch (10 g.) was dispersed by boiling  $\frac{1}{2}$  - 1 hour in oxygen-free water (2 litres). The dispersion was allowed to cool, filtered through glass wool to remove any undispersed granular material and stored in a nitrogen atmosphere. The concentration of the dispersion was determined by the method detailed in Section 2.

An aliquot of the dispersion (100 ml.) was heated slowly to 80°C in a 250 ml., three-necked flask fitted with a condenser, a stirrer and a gas-inlet tube through which nitrogen (oxygen-free) was passed. Complexing agent (1 ml.) was added and the dispersion allowed to cool slowly with constant stirring. With some of the more volatile hydrophobic reagents, more than 1 ml. was required in order to have a second liquid phase present; in order to provide a standard of comparison, thymol was added to one of the small-scale dispersions



dispersions. On cooling to room temperature, the dispersion was stored for three days. The resultant amylose complex was removed by centrifugation (15 minutes; 20,000 x g.). The amylopectin fraction was recovered by freeze-drying the supernatant solution after centrifugation. The yield of precipitate was estimated by subtraction of the weight of the freeze-dried product from the original weight of starch. Samples of the products were kept for characterisation.

Some of the amylose fractions were redissolved in boiling, oxygen-free water (100 ml.). On cooling to 80°C, hydrophobic precipitant (1 ml.) was added, and the whole was allowed to cool slowly to room temperature with constant stirring. After a period of 24 hours the precipitated amylose was removed by centrifugation (10 minutes; 1500 x g.). Prior to addition of the precipitant and after removal of the amylose complex, aliquots (3 x 1 ml.) of the solution were withdrawn for estimation of the concentration (see Section 2.). The yield of precipitate was thus readily obtained by calculation.

#### Characterisation of the Fractions.

The following characteristics were determined for the amylose fractions:- (1) iodine affinity, (2) limiting viscosity number in 0.15M potassium hydroxide at 25°C, (3)  $\beta$ -amylolysis limit and (4)  $\beta$  + Z-amylolysis limit. The amylopectin fractions were characterised with respect to iodine-affinity and  $\beta$ -amylolysis limit.

## RESULTS AND DISCUSSION.

The results of fractionation of a 0.5% potato starch dispersion by hydrophobic complexing agents are shown in Table 5. 3. The initial precipitates were obtained in extremely large yields (35-65%) and consequently were much less pure than the products of French et al (1963). The large yields may be attributed to the use of a high centrifugal force-field (20,000 x g.). However, it must be emphasised that centrifugation at speeds of 2000-3000 r.p.m. was totally ineffective in removing the amylose complexes from the dispersions. Experiments carried out under the dispersion conditions of French et al (i.e. 1% starch dispersion) showed that the amylose complexes could not be removed in an ordinary laboratory centrifuge even when the dispersion had been stored for a period of several weeks. In this context, the present findings are in direct contrast to the results of French et al. The small scale of the fractionations made the use of a Sharples Supercentrifuge impossible, but it is suggested that this method of centrifugation might be more suitable for large-scale separation of hydrophobic complexes of amylose. This suggestion is especially pertinent since the hydrophobic complexes were similar in consistency to the amylose-thymol complex.

The results of iodine-binding determinations indicate that the amylose content of the precipitates varied from 30 to 60%. It may be seen that where the precipitates were obtained in small yield, the amylose content of the precipitates was large. It would appear therefore /

therefore, that selective precipitation was occurring although a considerable amount of amylopectin was coprecipitated. The values for the limiting viscosity number of some of the complexes reflect the purity of the samples although it must be emphasised that, where large amounts of branched material are present, the viscosity of a solution is not a sensitive measure of the nature of the linear component in solution.

Whilst the amylopectin fractions were all obtained in small yield (30-65%), measurement of the iodine-affinity showed that they were all of very high purity (>99%). This may merely be a consequence of the low yield; nevertheless, where the yield was greatest, in the case of sample A, the amylopectin contained only 0.8% of amylose. The values for the  $\beta$ -amylolysis limits, shown in the last column of Table 3. 3, also indicate the high purity of these amylopectins.

Although the amylose complexes initially obtained were impure, recrystallisation of some of the samples with hydrophobic reagents, presented a vastly different picture, as shown in Table 3. 4. The yields, expressed as a percentage of the original hydrophobic complex, show that under the recrystallisation conditions (ca 0.1% concentration), precipitation of the amylose was much more selective. Also the reprecipitated complexes were readily removed in an ordinary laboratory centrifuge (1500 x g.). The recrystallised amyloses were, apart from the complex with 1,1,2,2-tetrachloroethane, considerably more pure than the products of French et al (1963). The results of iodine /

iodine-affinity determinations for some of the samples verify the high degree of purity indicated by the  $\beta$  + Z-amylolysis limits. In addition, the increase in limiting viscosity number also shows that branched material has been removed during the recrystallisation process.

The hydrophobic amylose complexes were formed readily on recrystallisation. In general, all the hydrophobic compounds examined compared favourably with thymol as starch fractionating agents. However, further investigation of the dispersion and centrifugation conditions is necessary.



TABLE 3.3.

Properties of the Starch Fractions.

Sample	Precipitant	Yield %	Iodine Affinity	Amylose %	$[\eta]$	Yield %	Iodine Affinity	Amylose %	$\beta$
A	Benzene	35	11.2	58	-	65	0.16	0.8	55
B	Fluorobenzene	55	7.1	37	275	45	0.10	0.5	55
C	Methyl cyclohexane	68	6.0	31	-	32	0.04	0.2	53
D	Cyclohexene	55	7.2	37	-	45	0.12	0.6	54
E	2,3-dimethylbutane	40	10.5	54	370	60	0.06	0.3	53
F	1,2-dichloroethane	45	10.1	52	-	55	0.14	0.7	54
G	1,1,1-trichloroethane	40	10.5	54	360	60	0.14	0.7	54
H	1,1,2-trichloroethane	40	10.6	54	-	60	0.07	0.35	53
I	1,1,2,2-tetrachloroethane	65	6.6	34	240	35	0.04	0.2	53
J	1,2-dichloropropane	40	10.7	55	-	60	0.15	0.8	54
K	Chloroform	48	8.9	46	330	52	0.06	0.3	54
L	Carbon tetrachloride	55	7.5	39	-	45	0.10	0.5	53
T	Thymol	55	7.2	37	250	45	0.02	0.1	53

Expressed as a percentage of the total starch.

\* Calculated from iodine affinity.

\* Percentage conversion into maltose under the action of pure  $\beta$ -amylase

TABLE 3.4.  
Properties of the Recrystallised Complexes.

Sample	Precipitant	Yield (%) $\dagger$	$\beta$ $\dagger$	$\beta$ $\dagger$	Iodine Affinity	Amylose (%) $\dagger\dagger$	$[\eta]$
B	Fluorobenzene	20	80	100	19.0	98	470
C	Methyl cyclohexane	32	76	98	-	-	-
G	1,1,1-trichloroethane	42	75	98	18.7	96	475
H	1,1,2-trichloroethane	50	80	99	-	-	-
I	1,1,2,3-tetrachloroethane	49	68	88	12.7	65	-
J	1,2-dichloropropane	54	74	97	-	-	480
K	Chloroform	39	78	97	17.7	91	-
L	Carbon tetrachloride	46	78	94	-	-	-
T	Thymol	39	79	98.5	18.9	97	490

$\dagger$  Expressed as a percentage of the initial complex.

$\dagger\dagger$  Percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase (2)  $\beta$ -amylase + Z-enzyme.

$\dagger\dagger$  Calculated from iodine affinity.

SECTION 4.

STUDIES ON MAIZE STARCH.

SECTION 4. I.

A Comparison of the Properties of Commercial and Laboratory-Extracted Maize Starches.

(a) Introduction.

The commercial preparation of starch often entails the use of deodorising and decolourising reagents (c.f. Radley, 1953) which are likely to cause degradation or structural modification. In the case of commercial potato starch, evidence has been accumulated in these laboratories that the amylose component is both degraded and has undergone structural modification (Cowie & Greenwood, 1957,c). However, there is no comparable information for maize starch. In the following studies, the properties of a commercial and a laboratory-prepared maize starch are compared.

(b) Experimental.

Materials.

The samples of commercial and laboratory-extracted maize starch were obtained by courtesy of Dr. S.A. Watson, of the Corn Products Company, Argo, Illinois. The samples were prepared as follows:-

Argo-Plant Starch. The starch slurry was obtained from the Dorr Clone units, filtered, washed with methanol and stored in methanol.

Fresh Maize Starch. Maize was picked from the field and shelled. The moisture content was 35%, indicating maturity of the starch but little drying of the kernels. After washing, to remove /



remove dirt, the maize (253g) was ground with water (280ml) and Pentasol (120ml) in a Waring Blendor at full speed for 1 minute. The mixture was screened on 40 mesh, then centrifuged for 2 minutes at 2500 r.p.m. The top layer (Pentasol, water and protein) was decanted and discarded. The starch centrifugate was reslurried in water (200 ml) and screened over nylon cloth (openings 44 x 53 microns). The residue was discarded. Pentasol (150ml) was added to the starch slurry which was again centrifuged; the supernatant was discarded. The starch centrifugate was filtered and washed, first with water, then with methanol. The resultant product was stored in methanol.

Prior to fractionation in these laboratories, the starches were shaken in toluene to remove any residual protein, stirred for several hours in boiling 85% aqueous methanol to remove fatty contaminants and pretreated (twice) with liquid ammonia. (See Section 2).

#### Characterisation and Fractionation of the Starches.

The starches were characterised with respect to their iodine affinities, and the amounts of nitrogen present.

Fractionation was achieved by two methods:-

(a) The starches were dispersed by boiling, in oxygen-free water, in a nitrogen atmosphere. Amylose was precipitated as the thymol complex and recrystallised twice with n-butanol. The amylopectin was recovered by freeze-drying the supernatant liquor after precipitation of the amylose with thymol.

(b) The starches were also fractionated by the method of Schoch (see Radley, 1953). Starch (5g; dry weight) was suspended /

suspended, in a litre flask fitted with a condenser and stirrer, in water (250ml) and n-amyl alcohol (17ml). The suspension was buffered at pH 6.1 - 6.2 by the addition of phosphate buffer (20%; 5ml). The flask was heated to 92°C, refluxed gently for 4 hours, then allowed to cool slowly and refrigerated for 24 hours, all with stirring. The amylose fraction which precipitated was separated using a high-speed centrifuge (18,000 r.p.m.). The supernatant was centrifuged once more. After extraction of the supernatant with ether and removal of excess ether on a rotary evaporator, the amylopectin was recovered by freeze-drying.

The crude amylose fraction was purified by recrystallisation (twice) with n-butanol, as follows:- The moist amylose fraction was added to boiling, oxygen-free water, to give an approx. 1% solution. Excess n-butanol was added and the solution was allowed to cool with stirring, refrigerated overnight and centrifuged (2000 r.p.m.).

The supernatant solutions, from both the first and the second recrystallisation with butanol, were extracted with ether and the polysaccharides recovered by freeze-drying.

#### Characterisation of the Fractions.

The amylose components were characterised with respect to their conversion into maltose under the action of (i) pure  $\beta$ -amylase and (ii)  $\beta$ -amylase + Z enzyme; limiting viscosity numbers were determined in M potassium hydroxide at 25°C.

The amylopectin samples were characterised with respect /

respect to  $\beta$ -amylolysis limit, average length of unit-chain and iodine affinity,

The intermediate samples isolated during the recrystallisation of the crude amylose obtained in the Schoch fractionation were characterised by their  $\beta$ -amylolysis limits and iodine affinities.

(c) Results and Discussion.

Properties of the Starches.

The properties of granular starches are shown in Table 4. 1.

TABLE 4. 1

Properties of the Granular Starches.

	Nitrogen %	Iodine Affinity	Amylose <sup>*</sup> %
Fresh Maize Starch	0.053	3.75	20
Argo-Plant Maize Starch	0.066	3.70	19.5

<sup>\*</sup> Calculated from Iodine Affinity.

The nitrogen values of 0.05 and 0.07% indicate that contaminating protein is negligible. The values for the iodine affinity are somewhat lower than values for maize starch quoted in the literature (e.g. Lansky, Kooi and Schoch, 1949). There is no significant difference, in iodine-binding capacity, between the laboratory and commercially prepared starches. The percentage amylose was calculated from the relation

$$\% \text{ Amylose} = \left( \frac{\text{Iodine Affinity}}{19.0} \right) \times 100.$$

The value of 19.0 for the iodine affinity of pure maize-amylose is an average value calculated from the results of Lansky et al (1949). /

(1949).

Properties of the Components.

Thymol-Fractionation.

The properties of the components obtained by fractionation using thymol are shown in Tables 4. 2 (Amyloses) and 4. 3 (Amylopectins).

TABLE 4. 2.

Properties of the Amyloses.

	$\beta$ i <sup>x</sup>	$\beta$ ii <sup>x</sup>	% Purity $\neq$	$[\eta]$ $\neq$
Fresh Maize Amylose	88	96	92	155
Argo Plant Amylose	80	88	76	125
<sup>x</sup> Percentage conversion into maltose under the action of (i) pure $\beta$ -amylase (ii) $\beta$ -amylase + Z-enzyme.				
$\neq$ Calculated from $\beta$ + Z amylolysis limit.				
$\neq$ Determined in M potassium hydroxide at 25°C				

TABLE 4. 3.

Properties of the Amylopectins.

	$\beta$ x	Chain Length	Iodine Affinity	Purity % $\neq$
Fresh Maize Amylopectin	58	21	0.46	97.5
Argo Plant Amylopectin	58	20	0.32	98.3
<sup>x</sup> Percentage conversion into maltose under the action of pure $\beta$ -amylase.				
$\neq$ Calculated from the iodine affinity.				

The amyloses are seen to be contaminated with amylopectin, since the combined action of  $\beta$  -amylase and Z-enzyme does not result in complete conversion into maltose. The viscosity results /



results indicate that the commercial amylose is smaller in size than the fresh-maize amylose. While this may be due to degradation of the commercial amylose, the smaller viscosity will also be caused, at least in part, by the presence of ca 24% of contaminating amylopectin.

The amylopectin samples are not as pure as those obtained from potato starch, using the same fractionation procedure. There are no significant differences in the properties of the amylopectins with regard to their chain-lengths and  $\beta$  - amylolysis limits. The chain-lengths are, however, shorter than most literature values for maize starch amylopectin. The gross characteristics of the amylopectin would not appear to be materially altered by the commercial extraction procedure. Although the amylopectins obtained were of reasonable purity, the thymol fractionation does not give pure amylose products. Since pure products are obtained when fractionating potato starch by this method, the inefficient fractionation of maize starch may be due to fundamental differences between the properties of maize starch and corn starch.

#### Amyl-Alcohol Fractionation.

The properties of the amyloses obtained by fractionation using amyl-alcohol are shown in Table 4. 4. The amyloses are purer than the corresponding amyloses obtained using thymol as an initial precipitant. The viscosity of the amylose from the commercial starch is again smaller than that from the laboratory-extracted maize starch. Since the amyloses are of comparable purity, the smaller viscosity must be caused by degradation of the amylose during the commercial-extraction process. A further /

further indication of degradation or of structural modification of the amylose is given by the fact that the commercial amylose has a lower  $\beta$ -amylolysis limit than the fresh-maize amylose; from the results in Section 6, the larger amylose molecule would be expected to possess the smaller  $\beta$ -amylolysis limit.

The properties of the polysaccharide isolated from the supernatant solutions, after recrystallisation of the amyloses, are shown in Table 4. 5. The results indicate that those substances are composed largely of amylopectin-type material together with about 5% of amylose. Although their properties vary slightly, these intermediate fractions are similar to the intermediate products found in the fractionation of potato starch (see Section 6).

TABLE 4. 4.

Properties of the Amyloses.

	$\beta$ (i) <sup>x</sup>	$\beta$ (ii) <sup>x</sup>	Purity % <sup>+</sup>	$[\eta]$ <sup>++</sup>
Fresh Corn Amylose	89	97	94	180
Argo Plant Amylose	87	97	94	140
<sup>x</sup> Percentage conversion into maltose under the action of (i) pure $\beta$ -amylase (ii) $\beta$ -amylase + Z-enzyme.				
<sup>+</sup> Calculated from the $\beta$ + Z amylolysis limit. <sup>++</sup> Determined in M potassium hydroxide at 25°C.				

TABLE 4. 5.

	$\beta$ <sup>≡</sup>	Supernatant 1		$\beta$ <sup>≡</sup>	Supernatant 2	
		Iodine Affinity	Amylose % <sup>+</sup>		Iodine Affinity	Amylose % <sup>+</sup>
Fresh Corn	67	0.09	4.8	63	1.35	7.0
Argo Plant	68	0.95	5.0	64	1.00	5.3

<sup>≡</sup> Percentage conversion into maltose under the action of pure  $\beta$ -amylase.  
<sup>+</sup> Calculated from iodine-affinity.

Table 4. 6 shows the characteristics of the amylopectins; these samples are not as pure as those obtained in the thymol-fractionation. There are no significant differences in the  $\beta$ -amylolysis limits and chain lengths of the two samples.

It may be generally concluded, from the results of both methods of fractionation, that the commercial process of extraction causes limited degradation of the amylose component of maize starch.

TABLE 4. 6.

Properties of the Amylopectins.

	$\beta$ x	Chain Length	Iodine Affinity	Purity % /-
Fresh Corn Amylopectin	61	21	0.60	96.9
Argo Plant Amylopectin	63	21	0.66	96.5
x Percentage conversion into maltose under the action of pure $\beta$ -amylase. /- Calculated from iodine-affinity.				

In addition, it must be noted that, although neither of the methods of fractionation employed gives pure starch components, the thymol-fractionation provides a relatively pure maize amylopectin while amylose of ca 97% purity is obtained if amyloalcohol is used as the initial precipitant.

SECTION 4. II.

STUDIES ON HIGH AMYLOSE-CONTENT MAIZE STARCHES.



(a) INTRODUCTION.

Because of the industrial potentialities of amylose, there has been, during the past decade, a considerable amount of research directed towards the production of starch containing a large proportion of amylose. This work has been restricted to the production of high amylose-content maize starch and as a result of genetic developments in maize, many new varieties, containing increased amounts of amylose, have been obtained. Zuber and coworkers (1958) have reported breeding inbred stock of maize containing up to 80%-amylose starch. This type of high-amylose maize is now more commonly referred to as amylomaize.

Wolff et al (1955) reported the results of structural studies on a starch containing about 50% of amylose. This starch had, not only a high amylose-content, but also had an amylopectin fraction of unique structure and properties, intermediate between the usual amylose and amylopectin fractions. Both the inner and outer branches of the amylopectin were reported to be longer than those of normal maize amylopectin.

High amylose-content starch has also been found to be present in wrinkled-seeded varieties of peas. The starch from this source contains ca 70% of amylose (Nielson and Gleason, 1945; Hilbert and MacMasters, 1946). Structural studies by Potter et al (1953) have indicated that the amylopectin component from this starch also has a much longer chain length (36 glucose units /

units) than the amylopectin from smooth-seeded peas (27 glucose units).

Examination by Greenwood and Thomson, (1960 and 1962) of the amylopectins both from wrinkled-seeded peas and from amylomaize, showed that the branched components had an apparent chain-length of 36 glucose units. However, on ultracentrifugation, the amylopectin samples were found to contain two components. Separation of these components by differential ultracentrifugation showed that one of the components was indistinguishable from a normal maize amylopectin, (chain-length 27 glucose units) while the other component possessed the properties of a short-chain amylose. In the above work, the amylopectins were obtained as products of a fractionation involving the precipitation of amylose from an aqueous dispersion of the starch; pretreatment with liquid ammonia was necessary to obtain complete dispersion.

Montgomery et al (1961) fractionated a series of eight inbred and two hybrid corn starches, with six levels of amylose content, into their respective amylose and amylopectin components, in order to compare amylomaize starch and its fractions with dent maize starch and its fractions. Fractionation was achieved by an extraction-sedimentation procedure developed by Montgomery and Senti (1958) for the fractionation of dent maize starch. Although this method is not dependent on complete dispersion of the starch, these authors claimed that pretreatment of undegraded dent maize starch, in certain hot organic solvents, altered the solution properties of the granule /

granule sufficiently to permit sharp separation of the amylose and amylopectin components. The above authors were unable to confirm Greenwood and Thomson's finding that the unusually long chain-length of the amylopectin was due to contamination by short-chain amylose. This observation is, perhaps, not surprising, since the two groups of workers used different samples of starch and different methods to fractionate the starches.

In the present work, two samples of amylomaize starch have been fractionated by the method of Greenwood and Thomson and by the method of Montgomery and Senti, in an attempt to decide whether the amylopectin from an amylomaize starch has, in fact, a longer chain length than normal amylopectin or whether this phenomenon is merely an artifact.

(b) Experimental.

Materials.

Two samples of commercial high-amylose-content corn starch were obtained through the courtesy of Dr. J. Goodwin of the Corn Industries Research Foundation, Washington, D.C. These were termed Amylon and Amylon 70 and contained, respectively, 50 and 70% of amylose. Prior to fractionation the starches were shaken with toluene to remove protein and stirred for several hours in boiling, 85% aqueous-methanol to remove fatty contaminants. Before fractionating using thymol, (see method (a) below) the starches were also pretreated (twice) with liquid ammonia.

Characterisation and Fractionation of the Starches.

The starches were characterised with respect to iodine affinity and the percentage nitrogen present. Fraction-

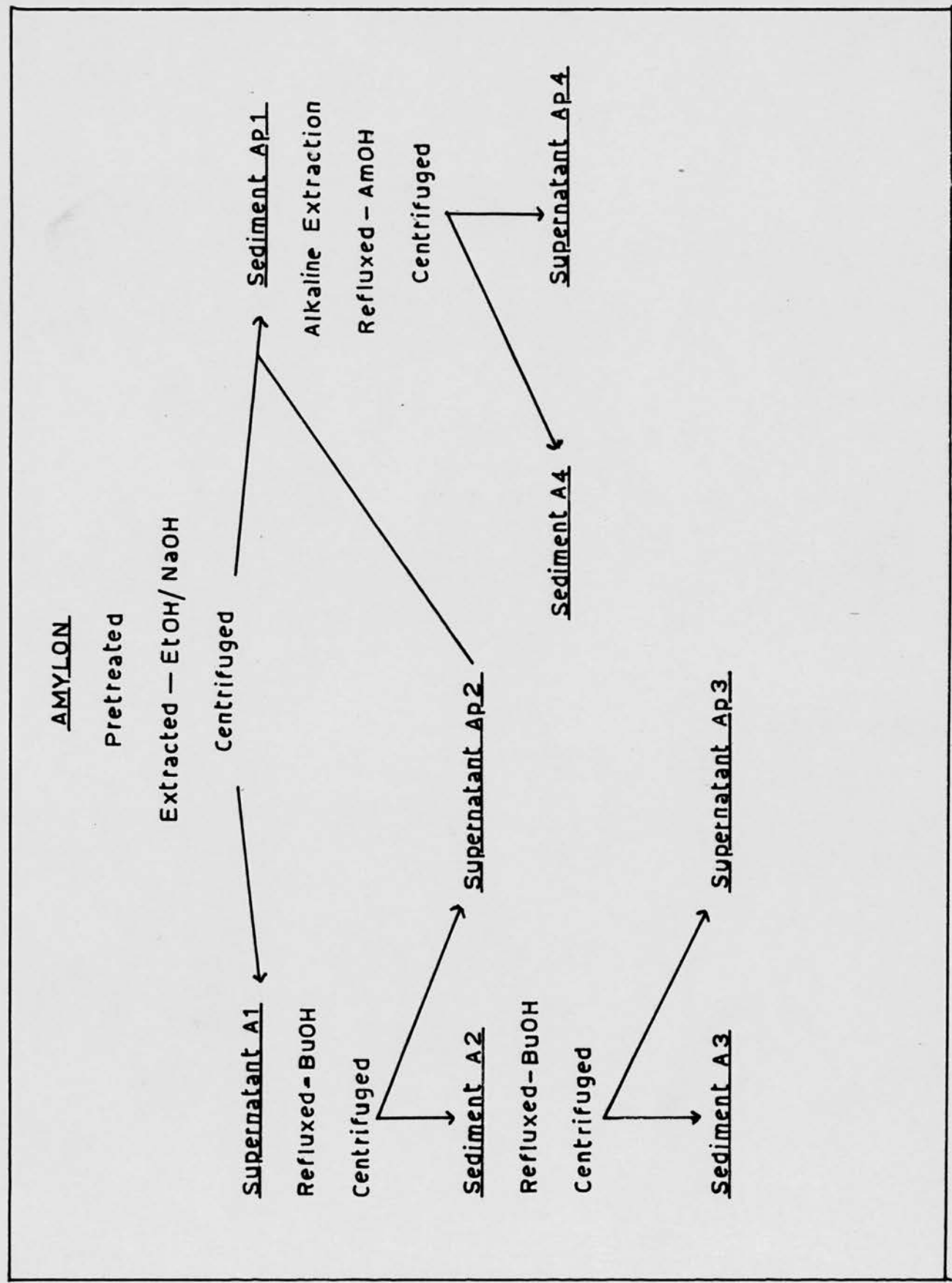
fractionation was achieved by two methods.

- (a) The starches were dispersed in boiling water, (nitrogen atmosphere) after heating for  $\frac{1}{2}$  - 1 hour. Amylose was precipitated as the thymol complex, separated using a Sharples Supercentrifuge, and recrystallised (twice) with n-butanol. The amylopectin was recovered by freeze drying the supernatant liquor after precipitation of the amylose with thymol.
- (b) The Amylon starch was also fractionated by the method of Montgomery and Senti (1961).

Amylon (20g; dry weight) was suspended in 400ml of a mixture of distilled water, n-butanol and glycerol (respectively 30%; 35% and 35%; w/w). The suspension was heated to 98°C and maintained at that temperature for 3½ hours. After cooling quickly to 30°C, in an ice-water bath, the suspension was stirred in ethanol (2 litres) for ten minutes. Three further extractions with ethanol were carried out, to ensure complete removal of the organic solvents. The washed starch was then weighed to estimate the ethanol content and slurried in sufficient additional ethanol to make a total solvent weight of 150g. The alcoholic starch slurry was added to 250ml. of sodium hydroxide (3N; deaerated; carbonate free) at 10°C. The mixture was stirred and kept at 10°C for  $\frac{1}{2}$  hour in a 5 litre, three-necked distilling flask. Dispersion at 10°C in 0.5N sodium hydroxide, containing 10 - 12% ethanol, was carried out by adding /



FIG.4.1



adding 1,200ml. of deaerated water containing phosphate buffer<sup>H</sup>, and shaking gently for 15 minutes in a nitrogen atmosphere. The alkaline dispersion was neutralised to pH 6.5 to 6.6 with hydrochloric acid (5N) added slowly from a burette. The mixture was sedimented at 3700g. for 30 minutes, yielding the precipitate Apl (See Fig. 4. 1). The supernatant solution contained the crude amylose fraction Al.

The supernatant containing the amylose was filtered through a sintered-glass filter (G3) refluxed with excess n-butanol for 30 minutes, and allowed to cool overnight. The amylose-butanol complex (A2) was separated using a laboratory centrifuge. Polysaccharide remaining in the supernatant liquor (Ap2) was precipitated by the addition of several volumes of ethanol, separated by filtration and combined with the sediment (Apl) from the first alkaline extraction. The amylose-butanol complex (A2) was recrystallised once more with n-butanol, yielding the major amylose fraction, (A3). The fraction Ap3, was recovered by freeze-drying the supernatant solution.

The total starch residual (Apl + Ap2) was covered with 1000ml. of deaerated water containing phosphate buffer at 25°C. Sodium hydroxide (100ml.; 5N; deaerated; carbonate free) was added, together with ethanol (50ml.) and the mixture was moderately /

---

\* The phosphate buffer contained potassium dihydrogen phosphate (50ml; 0.2M) and potassium hydroxide (17.8ml; 0.2M) and sufficient water to make a total volume of 200ml. For use, 1 ml. of buffer was diluted to 100ml.

moderately shaken for 30 minutes. The resultant dispersion was stored at 0 - 2°C for 24 hours, neutralised at 25°C, re-fluxed for 15 minutes with an excess of n-amyl alcohol and allowed to cool to 25°C. The amyl alcohol-amylose complex (A4) was removed by centrifugation.

The supernatant solution was diluted with an excess of ethanol (with stirring). The precipitate was collected by centrifugation and dissolved at once in 400ml. of buffered water by boiling for 15 minutes. On cooling, the solution was centrifuged to remove the remaining traces of protein and other extraneous material. To remove salts, the amylopectin solution was dialysed, at room temperature, against 10 volumes of distilled water containing 5% ethanol, with several changes of dialysing solvent. The amylopectin (Ap4) was recovered by freeze-drying.

Characterisation of the Fractions.

Thymol Fractionation.

The amylose fractions obtained by fractionation using thymol were characterised with respect to their conversion into maltose under the action of (i) pure  $\beta$ -amylase and (ii)  $\beta$ -amylase + Z-enzyme. Both samples of amylose obtained were insoluble in water, even with heating. Solutions for enzymic assays were therefore prepared by dissolving the amylose-butanol complexes in sodium hydroxide (ca 0.2M) and neutralising with hydrochloric acid (2N). The limiting viscosity numbers of the amyloses were determined in M potassium hydroxide at 25°C.

The amylopectin samples were characterised with respect to  $\beta$ -amylolysis limit, average length of unit-chain and iodine affinity.

To estimate the reducing powers of the amylopectins, aliquots (3ml.) of aqueous solutions were heated for 15 minutes in the presence of potassium ferricyanide (2.5ml.; 0.05M) and sodium carbonate (2.5ml. 0.2M). (See Section 2). On cooling, the solutions were acidified with sulphuric acid (5N; 5ml.) and titrated with ceric sulphate (0.01N). Similar determinations were carried out for a pure sample of potato amylopectin and for the amylo maize amyloses. The reducing powers were expressed as equivalents of ceric sulphate/g. of polysaccharide.

Both amylopectin samples were examined on an analytical scale using a Spinco ultracentrifuge (Model E) (0.3% concentration in 0.2M potassium hydroxide). Differential ultracentrifugation was carried out by spinning an aqueous solution (0.3% concentration; 100ml.) in a preparative rotor, at 44,000 r.p.m. for 1 hour. The resultant precipitate was re-dissolved in water and filtered through a sintered-glass filter (G 4). The polysaccharide was recovered by freeze-drying the solution. The polysaccharide present in the supernatant solution after ultracentrifugation was also recovered by freeze-drying.

Differential ultracentrifugation was also carried out following the procedure of Greenwood and Thomson (1962) i.e. an aqueous solution (0.2% concentration) was spun for 2 hours (twice) at 44,000 r.p.m. The products were again obtained by freeze-drying. The amylopectin subfractions were characterised with respect to  $\beta$ -amylolysis limit, chain-length and both the colour and wavelength of maximum absorption of a solution of the polysaccharide stained with iodine.

Extraction /



### Extraction-Sedimentation Fractionation.

The amylose-type fractions were characterised with respect to iodine-affinity,  $\beta$ -and  $\beta + Z$  amylolysis limits and limiting viscosity number in M potassium hydroxide at 25°C. The characteristics determined for the amylopectin-type fractions were the iodine-affinity,  $\beta$ -amylolysis limit and the chain-length.

The main amylopectin fraction (A4) was further subfractionated as follows:- Contaminating amylose was precipitated by the addition of iodine (5ml.; 0.2M; in 2M potassium iodide), at room temperature, to an aqueous solution (75ml.; 0.2%) of the amylopectin. The resultant solution was refrigerated (4°C) for 48 hours. Centrifugation at 3500 r.p.m. yielded a precipitate which was redissolved in water. The iodine, present in both this solution and the supernatant from the centrifugation, was removed by the addition of sodium thio-sulphate. Both solutions were then dialysed for several days against distilled water (20 volumes) with frequent changes of dialysing solvent.

The polysaccharides were recovered by freeze-drying and characterised in the same manner as the other fractions.

(c) Results and Discussion.

Properties of the Starches.

The protein-content of the Amylon and Amylon 70 was 0.38 and 0.44% respectively. This was regarded as negligible. Estimation of the iodine-binding power of the starches was carried out on defatted samples. The resultant curves (Fig. 4. 2) were of unusual shape, in that the binding of iodine occurred more slowly and terminated at a higher free-iodine concentration than is generally found for maize or potato starch. Since amylose generally binds iodine rapidly, at low concentrations of iodine, the gradual absorption found in these amylo maize starches could be attributed to the presence of short-chain amylose. Extrapolation of the linear part of the curves to zero free-iodine concentration, to give the iodine-affinity, produced values (7.2 and 8.6) which correspond to the presence of respectively 38 and 45% of amylose in the Amylon and Amylon 70. These values are much lower than the commercially-quoted values; the reason for this is not understood.

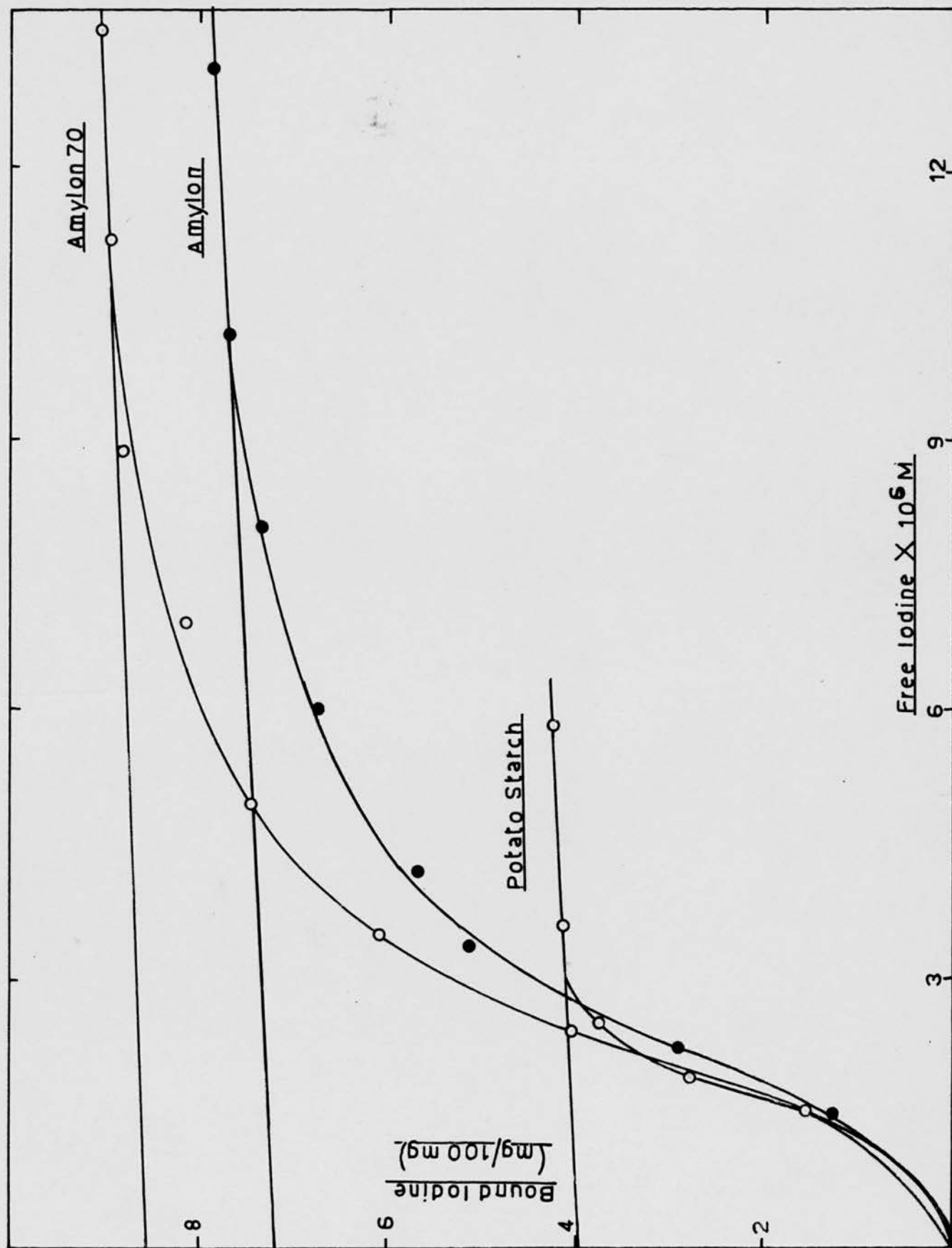
Attempts to use the special technique of Deatherage et al (1955) were unsuccessful, due to the insolubility of the amylo maize starches in calcium chloride solution.

Properties of the Fractions.

Thymol-Fractionation.

The properties of the products of thymol-fractionation are shown in Table 4. 7. The amyloses, even after two recrystallisations with n-butanol, were impure, as they were incompletely /

FIG. 4.2.



incompletely converted into maltose under the combined action of  $\beta$ -amylase and Z-enzyme. The  $\beta + Z$  amylolysis limits correspond to the presence of ca 12 and 20% respectively of amylopectin in the Amylon and Amylon 70 amyloses. The values for the limiting viscosity number indicate that the amylose fraction in both starches is of relatively short chain-length. Though these values will be affected by the contaminating amylopectin, they are in favourable agreement with those quoted by Anderson et al (1963) in their studies on the fractionation of amylomaize starches. Since the total amylose fraction is of short chain-length and since the starches contain large amounts of amylose, it is not unreasonable to suggest that very short-chain amylose will be present in greater quantity than is usual for maize starch. This suggestion is in accord with the earlier indications from iodine-binding determinations.

In agreement with earlier workers (e.g. Greenwood and Thomson, 1960) the amylopectin fraction has a longer chain-length than is usually found for maize amylopectin. The calculated values for the internal chain-length are only slightly higher than the value of 8 - 9 glucose units usually quoted for potato amylopectin. However, the values of 35 and 36 glucose units for the chain-lengths are associated with  $\beta$ -amylolysis limits of ca 65% and iodine affinities indicating the presence of 7 - 8% of contaminating amylose. While these figures could be accounted for on the basis of a long-chain amylopectin, a further indication of contaminating amylose is provided by the values /



TABLE 4.7.

AmylosesProperties of the Fractions.Amylopectins

Sample	# $\beta$ 1	# $\beta$ 2	[ $\eta$ ]	# $\beta$ 1	Chain Length	Internal Chain Length	Iodine Affinity	Amylose (%)	Reducing Power X
Amylon	75	94	100	65	55	10	1.4	7.4	9.7
Amylon 70	70	90	100	66	56	10	1.6	8.4	16.7

TABLE 4.8.

Properties of Subfractions of the Amylopectin.SedimentSupernatant

Sample	Centrifugation Time	Yield (%)	# $\beta$ 1	Chain Length	Iodine Stain	$\lambda$ max. ( $\mu$ )	Yield (%)	# $\beta$ 1	Iodine Stain	$\lambda$ max. ( $\mu$ )
Amylon	1 hour	70	56	27	Red	560	30	76	Blue	580
Amylon 70	1 hour	75	57	26	Red	560	25	81	Blue	580
Amylon	2 hours	80	57	27	Red	560	20	79	Blue	580
Amylon 70	2 hours	80	57	28	Red	560	20	88	Blue	585

# Percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase (2)  $\beta$ -amylase + Z enzyme.

/ Calculated from iodine affinity.

X Expressed as equivalents of ceric sulphate  $\times 10^4$ /gm. of amylopectin.

// Calculated from {chain length - (chain length  $\times$  limit + 2.5)}

values for the reducing power. The results shown in Table 4. 7 are much higher than those found for a pure potato-amylopectin ( $1.6 \times 10^{-4}$  equiv./g.) and are intermediate between this value and those found ( $25$  and  $20 \times 10^{-4}$  equiv./g. respectively) for the Amylon and Amylon 70 amylose samples. Since, theoretically, each amylopectin molecule contains only one reducing group, a pure amylopectin should, effectively, have no reducing power. The small, finite value found for the potato-amylopectin could be caused by a small amount (0.7%) of amylose; in addition, it is possible for hydrolysis to occur during heating of the amylopectin. The enhanced reducing-power of the amylopectins, taken in conjunction with the high -amylolysis limits and iodine-affinities, can lead only to the conclusion that these amylopectins are contaminated with amylose.

Ultracentrifugal examination showed the unambiguous presence of two components in only one of the amylopectin samples. The major peak observed in this case, (Amylon amylopectin) was relatively slow moving and showed a wide distribution of molecular size. The single peak observed for the Amylon 70 amylopectin was similarly shaped. Since the starch samples were commercial ones, there is a high probability that the apparent small size of the amylopectin component was due to degradation.

Ultracentrifugal separation, on a preparative scale, of the two substances observed in the Amylon amylopectin and similar treatment of the Amylon 70 amylopectin gave the results shown in Table 4. 8. The polysaccharides which sedimented after centrifugation for 1 hour at 44,000 r.p.m. (0.3% concentration /

concentration) comprised ca 75% of the original amylopectin and possessed  $\beta$ -amylolysis limits and chain-lengths corresponding to normal amylopectins. Further, the colour and wavelength characteristics, produced on staining solutions of the sedimented polysaccharides with iodine, indicate the absence of any amylose-type material. The results obtained after centrifugation under the conditions of Greenwood and Thomson (1962) are similar, although the yields are higher due to the longer sedimentation times.

The characteristics of the polysaccharides remaining in the supernatant solutions after centrifugation, are those of an amylose. The  $\beta$ -amylolysis limits vary from 76-88%. These results are at variance with those of Greenwood and Thomson (1960 and 1962), who quote  $\beta$ -amylolysis limits of 100%. This discrepancy may be due to natural difference between the samples of starch used. On the other hand, the incomplete conversion into maltose found for the samples in the present work, may be due to structural modification of the amylose during commercial extraction of the starch from the maize. The shift, in wavelength of maximum absorption of the blue amylose-iodine complex, to shorter wavelengths (580 - 585  $m\mu$ ) has also been observed by Wolff et al (1955).

#### Extraction-Sedimentation Fractionation.

The properties of the starch fractions obtained by the fractionation procedure of Montgomery and Senti are shown in Table 4. 9. The amylose fractions were impure, as indicated both by the results from iodine-binding determinations and by the incomplete conversion into maltose by a mixture of  $\beta$ -amylase and /

and Z-enzyme. The final amylose product (A3) was similar to that obtained in the thymol-fractionation. The secondary amylose fraction (A4), obtained only in small yield, was also contaminated by amylopectin. While the results in Table 4. 9 indicate that recrystallisation with n-butanol was successful in increasing the purity of the amylose, the final product contained ca 10% of amylopectin. In the case of the extraction-sedimentation fractionation, the inefficiency of fractionation could be explained on the basis of incomplete dispersion and molecular disentanglement of the starch components; such a hypothesis would, however, be untenable in the case of the thymol-fractionation, where pretreatment with liquid ammonia gave complete dispersion of the starches in boiling water after heating for  $\frac{1}{2}$  to 1 hour. Since two recrystallisations with n-butanol are generally sufficient to purify a potato amylose, the amylose products obtained from amylomaize starches would appear to be radically different in character.

It is of interest to note that the product remaining in solution after recrystallisation with n-butanol (Ap2 and Ap3) resembled normal maize-amylopectin more closely than did the main amylopectin fraction (A4). <sup>? Ap<sub>4</sub></sup>

The amylopectin (A4)<sup>?</sup> was similar in character to the amylopectin obtained using thymol as an amylose precipitant. In agreement with the results of Montgomery and Senti (1961) the chain-length was longer (35 glucose units) than that of normal maize amylopectin; in addition, the amylopectin bound

a /



TABLE 4. 9.

Properties of the Starch Fractions.

Sample	Iodine Affinity	Amylose (%) $\dagger$	$\beta_1$	$\beta_2$	$[\eta]$	Chain Length
A1	-	-	63	69	50	-
A2	12.0	63	70	76	70	-
A3	17.8	89	83	94	100	-
A4	13	69	75	82	75	-
Ap2	0.2	1.0	57	-	-	-
Ap3	0.3	1.5	60	-	-	30
Ap4	1.8	9.5	65	-	-	35

TABLE 4. 10.

Sample	Yield (%)	Iodine Affinity	Amylose (%) $\dagger$	$\lambda_{\text{max.}}$ ( $m\mu$ )	$\beta_1$	$\beta_2$	Chain Length
Supernatant	85	0.4	2.0	560	59	-	28
Sediment	15	-	-	580	100	100	-

$\dagger$  Percentage conversion into maltose under the action of (1) pure  $\beta$ -amylase (2)  $\beta$ -amylase + Z-enzyme.

$\dagger$  Calculated from iodine affinity.

a considerable amount of iodine. However, on subfractionation of this component with iodine, the results shown in Table 4. 10 were obtained. These results, in confirmation of the earlier results from ultracentrifugation, indicate that the high-iodine binding power and the unusually long chain-length were indeed caused by the presence of contaminating amylose. The yield of contaminant (15%) was, in this instance, lower than that found using ultracentrifugal separation. The  $\beta$ -amylolysis limit (100%) was in agreement with the results of Greenwood and Thomson (1960 and 1962) and indicated that the amylose-contaminant was of short chain-length. The properties of the remaining amylopectin were again found to be those of a normal maize-starch amylopectin. The earlier work by Greenwood and Thomson, in these laboratories, has thus been confirmed.

In general, the results indicate that the extraction-sedimentation procedure is not an efficient method of fractionating high amylose-content maize starches; the same criticism must be applied to the thymol-fractionation. It must, however, be emphasised that the criticism may not be generally applicable to amylomaize starches since, in this work, the fractionation was complicated by the fact that the only samples of amylomaize starches available were ones obtained from the maize by a commercial extraction procedure.

SECTION 5.

An Investigation of the Starch from  
the Fruit of the Potato, Solanum Tuberosum.

## INTRODUCTION

Although nearly every variety of the potato, Solanum Tuberosum, produces flowers, pollen sterility restricts the number which actually bear fruit when the plant reaches maturity. The fruit - the so-called potato ball, potato apple or potato berry - is an essentially spherical, brown or purplish green berry. In common with many other fruits, the potato berry contains starch, but this has not been investigated in detail before. Moreover, the granules of this starch possess more than usually prominent concentric lamellations, (Figs. 5. 1 and 5. 2) - a characteristic which was first recorded in a monograph by Griffiths (1892). The only subsequent reference to this starch is a photomicrograph of the granules shown in a recent review by Schoch (1962).

While recent use of the electron microscope and radioactive tracer studies has generally led to greater knowledge of granular structure and the method of deposition of starch in the granule, the external lamellar markings are not yet completely understood. These markings are normally fairly prominent in potato starches, being some  $1 - 2\mu$  wide and some  $3 - 4\mu$  apart. While their appearance must be /

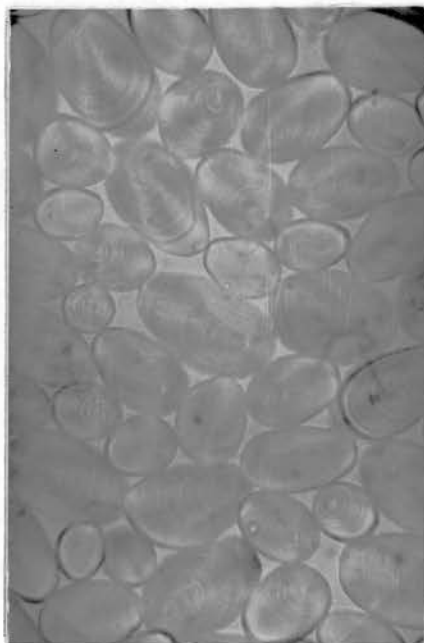
FIG. 5.1

SAMPLE M109/3 (MAG. X 400)

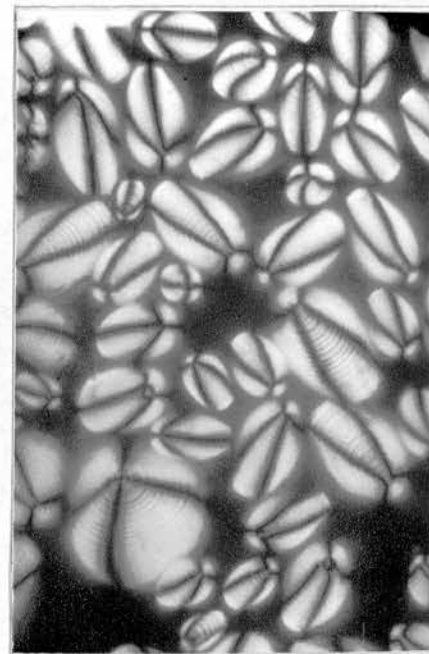
Tuber Starch

Ordinary Light

Berry Starch



Polarised Light



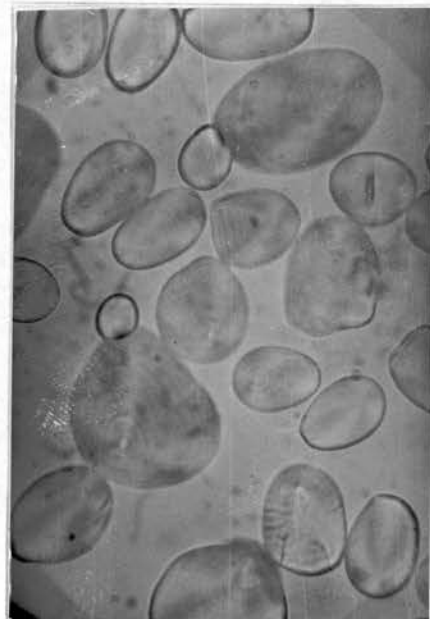


## FIG.5.2.

PENTLAND DELL

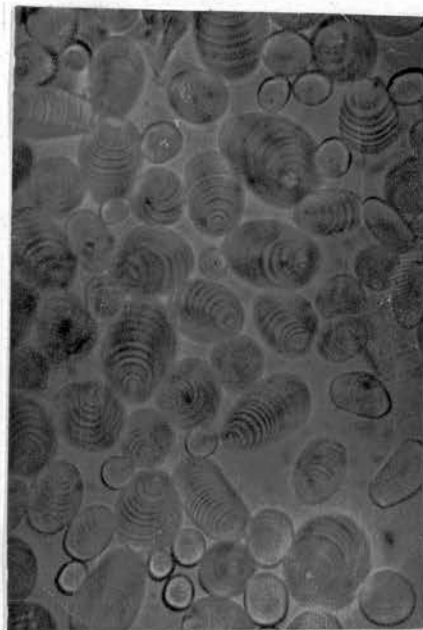
(MAG. X 400)

Tubet Starch

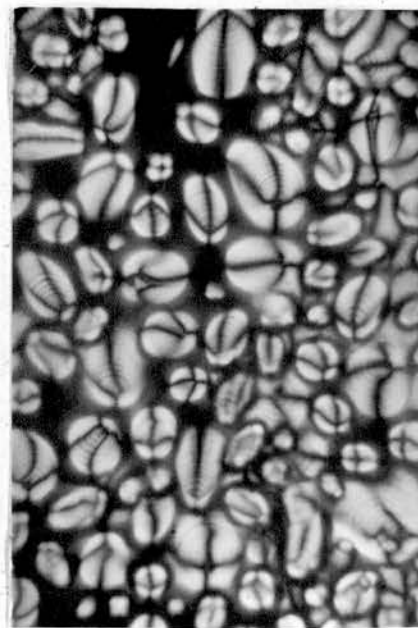
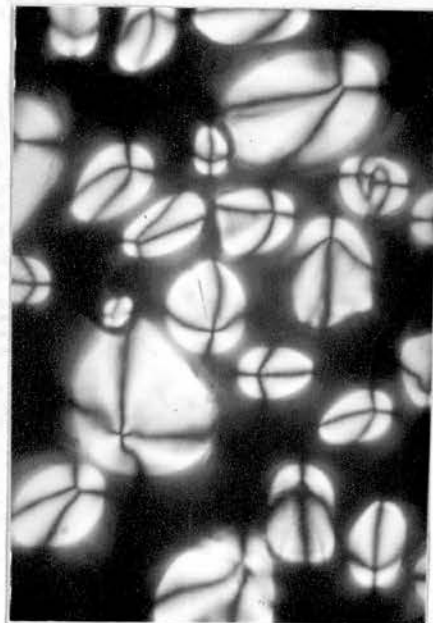


Ordinary Light

Berry Starch



Polarised Light



be caused by differences in refractive index, the factors governing periodic deposition of material during growth are more obscure. The researches of Buttrose (1962) show that, unlike barley and wheat starches, shell formation in potato starch granules is controlled by an endogenous rhythm unrelated to photosynthesis; Badenhuisen and Dutton (1956) have indicated that there is evidence of irregular translocation of sugars and starch deposition in the tuber itself. Electron microscopy studies on acid-treated potato starch granules have shown the granular shells to consist of some 50 - 100 submicroscopic lamellae. To account for these, Frey-Wyssling and Buttrose (1961) suggested the imposition of a short term (ca. 2 hours) endogenous rhythm on the longer rhythm mentioned above.

In the case of granules from the potato berries, it may well be that the increased prominence of the rings is connected with the fact that the berries are nearer the source of starch precursor. Further, since they grow above the ground the berries are more likely to be affected by an externally imposed starch deposition rhythm such as one dependant on a day-night alternation (c.f. wheat and barley). In this context it would be interesting and perhaps profitable to /

to determine whether such pronounced lamellations are produced in potato berries which have been grown under constant environmental conditions.

In view of the possible interest with regard to the problem of biosynthesis and granular architecture and in the hope that pronounced granular lamellations might run parallel with other physical and chemical differences in the properties of the starches and their components, starch granules were isolated from the fruits of several potato varieties in quantities sufficient for detailed investigation. Studies have been made of the properties of the granules and the products of fractionation and those have been compared with those of the corresponding tuber starches.

## EXPERIMENTAL

### Materials

The samples of potato berries were obtained from the Scottish Plant Breeding Station, Roslin, Midlothian, through the courtesy of Dr. W. Black. Three varieties of potato were chosen, primarily for the extent to which they bore fruit; these were two commercial varieties, - "Dr. MacIntosh" and "Pentland Dell" - and a Mexican blight-resistant strain of *S. Tuberosum*, sub species *Andigena*. The potato berries were collected from the plants in late August, 1963, when they were 2 - 2.5 cm. in diameter.

The starch content of the berries was estimated by extraction with perchloric acid followed by colorimetric estimation of the resultant extracts. (See Section 2). Since the internal structure of the berries was in many ways similar to that of the tomato and starch was manifestly present only in a layer between the outer cortex and the central seed-bearing area, a sample was chosen as near as possible representative of the whole berry. This was achieved by slicing the berry and taking a central section of the slice. Determinations were made in duplicate, giving results agreeing to <sup>+</sup> - 5%.

The starch was isolated from the plant material by the method indicated in Section 2.

### Characterisation of the Granules.

The following estimations were carried out as detailed in Section 2

- (a) Percentage nitrogen.
- (b) Percentage phosphorus.
- (c) Gelatinisation temperatures.
- (d) Percentage amylose.
- (e) Granular susceptibility to acid attack.

The method used was essentially that of Buttrose (1963). Samples of starch (100 - 150 mg; vacuum dried at 30°C) were treated, in 10 ml. centrifuge tubes, with hydrochloric acid (10 ml; 2.2 N) for period varying from  $\frac{1}{2}$  day to 5 days. The residues were washed several times with distilled water, centrifuged, and dried as before. Extent of attack was expressed as a percentage loss of weight.

- (f) Granular susceptibility to amylolytic attack.

Portions of non dried granules were treated with barley  $\alpha$ - and  $\beta$ - amylases (provided by Mr. A.W. MacGregor) in 40 ml. centrifuge tubes. The digests contained starch (ca 500 mg.), phosphate buffer (4 ml; pH 5.5) and enzyme solution (1 ml) and were maintained at 50°C for a period of about 50 hours. At appropriate time intervals the solutions were centrifuged and the soluble carbohydrate material /



material present in the supernatant was estimated by the phenol-sulphuric acid method. For this purpose, samples ( $\frac{1}{40}$  ml.) were withdrawn using a micropipette, while the optical densities of the final solutions were determined on an Eel photo-electric colorimeter using filter No. 623, with maximum transmission at 490 m $\mu$ .

#### Fractionation of the Granules.

Prior to fractionation, the starches were exhaustively defatted (See Section 2). Starch granules were dispersed directly in aqueous solution by boiling under reflux for  $\frac{1}{2}$  hour in a nitrogen atmosphere. Amyloses were precipitated as the thymol complex and then recrystallised as the butanol complex. Amylopectins were obtained by freeze drying the supernatant liquors after removal of the amylose.

#### Characterisation of the Amyloses.

The extents of conversion of the amylose samples into maltose under (i) the action of pure  $\beta$ -amylase and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme were measured. Limiting viscosity numbers were measured in M potassium hydroxide at 25°C using a modified Ubbelohde viscometer (See Section 2).

Characterisation /

Characterisation of the Amylopectins.

Measurement of iodine affinity to estimate the purity of the samples and measurement of the  $\beta$ -amylolysis limit were carried out as above. Average lengths of unit chain were calculated from potentiometric determinations of the amount of formic acid liberated on oxidation with sodium metaperiodate at 4° C as described in Section 2. The weight-average molecular weights as determined by light-scattering in 0.05 M potassium chloride were supplied by Mr. R. Geddes.

## Results and Discussion.

### Properties of the granular starches.

Since the characteristics of potato starch granules have been found to depend on the size of the granule and the maturity of the plant source (Section 6), it must be emphasised that only general comparisons can be made between the berry and tuber starches. The granular properties are listed in Table 5, 1. showing that the nitrogen and phosphorus contents of the two types are similar, but the average gelatinisation temperatures differ. These differences are better illustrated by the gelatinisation curves in Fig. 5, 3. One of the variables governing the complicated phenomenon of gelatinisation is the granular size - large granules being more easily gelatinised than small ones. Thus the above differences were resolved when microscopic examination indicated that the granules of the tuber starches were, in two samples, significantly smaller. Thus differences in gelatinisation behaviour must be due largely to differences in granular size rather than to the relatively small variations in apparent amylose content.

### Granular Structure.

In an attempt to find out whether the more pronounced lamellations on the berry starches reflected any changes in the organisation of the granules, the susceptibility of these starches to attack by acid and amylolytic enzymes was studied. A graph, typical of the effect of erosion by 2.2 N /

TABLE 5. 1

Properties of Starch Granules from the Tuber and Berry of the Potato.

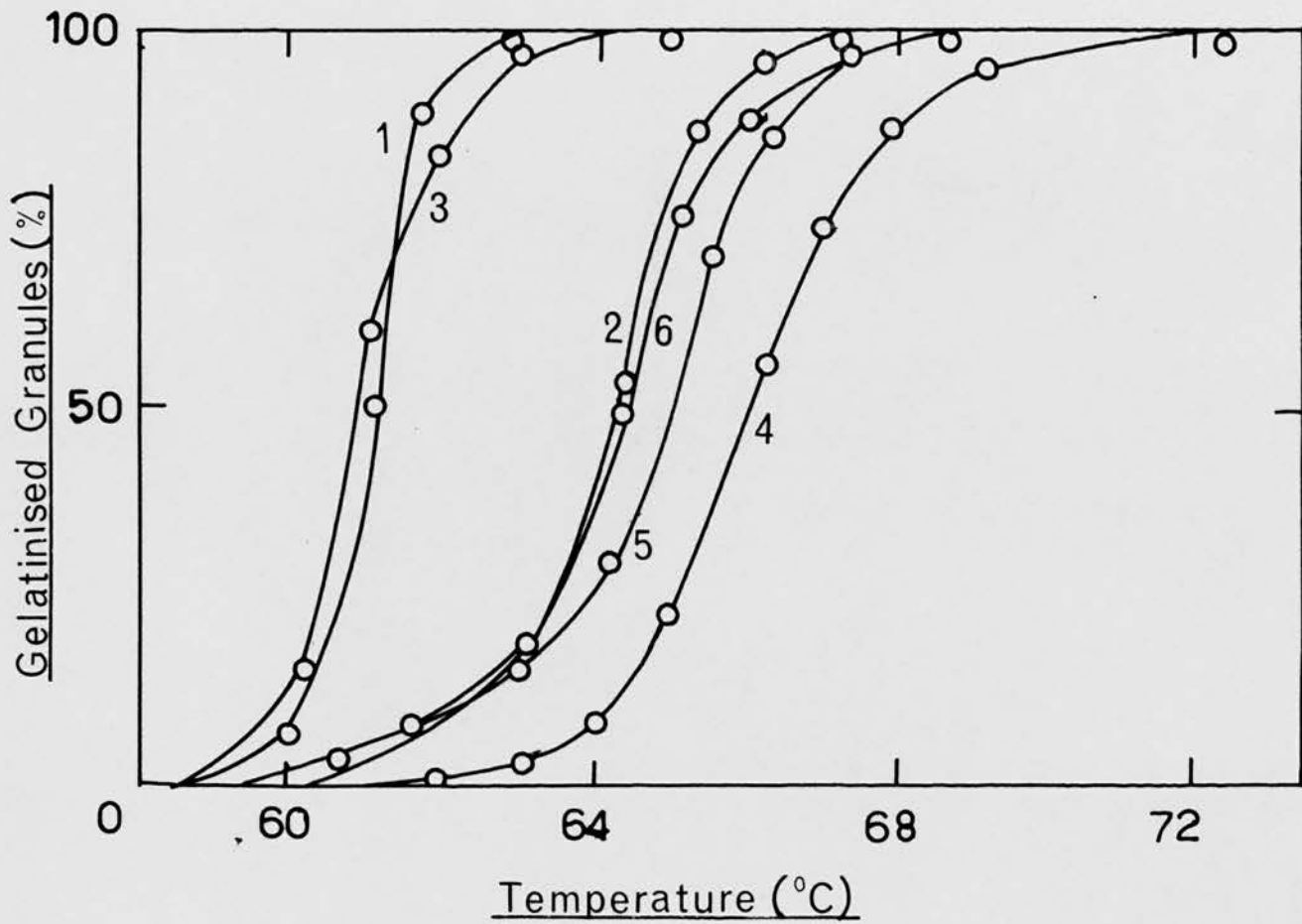
Variety	Starch source	Starch (%)	Nitrogen (%)	Phosphorus (%)	Av. gelatinization temp. (°C)	Iodine affinity <sup>1)</sup>	Amylose (%) <sup>2)</sup>
Dr. MacIntosh	Tuber	-	0.005	0.042	61	4.0	20.5
Dr. MacIntosh	Berry	10	0.007	0.031	64	3.6	18.5
Pentland Dell	Tuber	-	0.004	0.051	61	4.0	20.5
Pentland Dell	Berry	13	0.008	0.054	66	3.8	19.5
Mexican strain	Tuber	-	0.007	0.061	65	3.5	18.0
Mexican strain	Berry	11	0.004	0.042	64	3.4	17.5

1) Expressed as mg. I<sub>2</sub> bound per 100 mg. starch

2) Calculated from iodine affinity.

FIG. 5.3

- 107a -



Dr. MacIntosh 1, tuber ; 2, berry .

Pentland Dell 3, tuber ; 4, berry .

Mexican strain 5, tuber ; 6, berry .



2.2 N hydrochloric acid at 35°C, is shown in Fig. 5, 4, the rate of solubilisation being similar to that found by Buttrose (1963), for his sample of potato starch.

However, no appreciable differences in the rates of erosion between the berry and tuber starches were found. (Table 5, 2)

TABLE 5, 2.

Acid Attack on Granules.

M109/3

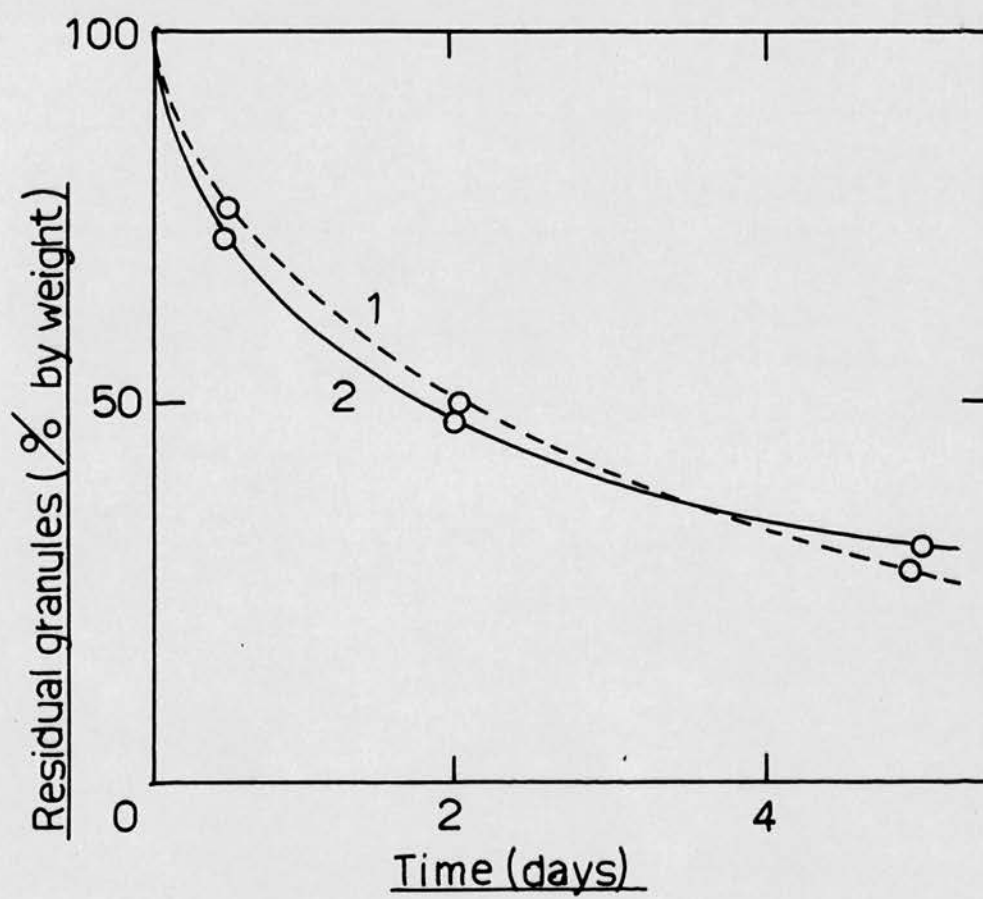
Dr. MacIntosh

Tuber		Berry		Tuber		Berry	
Time (Days)	Residual Weight (%)	Time (Days)	Residual Weight (%)	Time (Days)	Residual Weight (%)	Time (Days)	Residual Weight (%)
1/2	73	1/2	75	1	64	1	69
2	49	2	50	2	46	2	50
5	32	5	29	5	26	5	30

This result is perhaps not unexpected, as the ease with which hydrogen ions must be able to permeate the granular structure implies that only gross differences would affect the rate of attack. Microscopic examination showed that both berry and tuber starches were extensively damaged; in many cases the granules were completely disintegrated. Loss of birefringence was proportional to the amount of damage in the individual granule.

Differences in granular susceptibility became apparent when the granules were treated with a mixture of /

FIG. 5.4



of  $\alpha$ - and  $\beta$ -amylases from barley. Fig. 5.5. shows that, on a qualitative basis, the berry starches were more readily attacked. The decrease in rate of attack may merely reflect a loss of enzymic activity, since little is known about the stability of  $\alpha$ - and  $\beta$ - amylases at 50°C, though they are most active at this temperature. Should this be the case, the initial differences in extent of attack, (the conditions being otherwise identical) can be explained only in terms of ease of enzymic degradation of the structures of the starches concerned. Microscopically, erosion and enlargement of the hilum was evident in most of the granules, although few of them appeared to be completely destroyed. This conclusion only partially agrees with Leach and Schoch (1961) who, on attacking potato starch granules with bacterial  $\alpha$ -amylase observed that "potato starch showed no evidence of granule erosion or decrease of birefringence even after 56% solubilisation."; these authors further observed that the starch was solubilised by attack on individual granules and that the enzyme showed no preference for either the large or the small granules. The iodine affinity of the residual starches after enzymic treatment was substantially the same, (Table 5, 3) but it should be noted that the extent of solubilisation was limited.

FIG. 5.5



TABLE 5.3.

Iodine Affinities before and after Enzymic Treatment.

	<u>Before</u> <u>Enzymic Treatment</u>	<u>After</u> <u>Enzymic Treatment</u>
M109/3 Berry	3.4	3.35
M109/3 Tuber	3.5	3.45
Dr. MacIntosh Berry	3.6	3.5
Dr. MacIntosh Tuber	4.0	4.0

The results would indicate that there may be a difference, though not a radical one, between the berry and tuber starches. The more pronounced lamellations on the berry starches would not appear, therefore, to be due to gross differences in granular organisation and if there are more subtle differences, suitable techniques for their study do not, at the present time, exist. However, before condemning further examination of these starches to the indeterminate future, it might be profitable to assess the degree of crystallinity and also to subject the granules to electron - microscopic studies.



Properties of the starch components.

The properties of the amylose components obtained by fractionating the granular starches are shown in Table 5.4.

TABLE 5. 4.

Properties of Amylose Components from Starches

Variety	Starch source	$\beta$ -Limit $\#$ ) (i)	$\beta$ -Limit $\#$ ) (ii)	$[\eta]$	D.P. <sup>7)</sup>
Dr. MacIntosh	Tuber	75	101	540	4000
Dr. MacIntosh	Berry	85	99	450	3300
Pentland Dell	Tuber	85	99	390	2900
Pentland Dell	Berry	88	98	380	2800
Mexican Strain	Tuber	80	101	470	3500
Mexican strain	Berry	84	100	420	3100
$\#$ ) Conversion into maltose under the action of (i) pure $\beta$ -amylase, and (ii) $\beta$ -amylase and Z-enzyme. <sup>7)</sup> Degree of polymerization; calculated from $\overline{D.P.} = 7.4 \times$					

Values are given for the percentage conversion into maltose under (i) the action of pure  $\beta$  -amylase and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme. For all samples, degradation by the pure  $\beta$ -amylase was incomplete, although no contaminating amylopectin was present, as shown by the complete conversion into maltose on treatment with the mixture of  $\beta$ -and Z-enzymes. In that they contain a "barrier" to the action of  $\beta$ -amylase, these amyloses are no different /

different from other amyloses obtained from a variety of sources. Values for the degree of polymerisation shown in Table 5, 4 and calculated from the observed limiting viscosity numbers, are not thought to be exact. They should, however, be of the correct order of magnitude and certainly provide a useful measure of the relative sizes of the amylose molecules. The values for the berry amyloses were smaller than those for the corresponding tuber samples.

The purity,  $\beta$ -amylolysis limit, average length of unit chain and the calculated internal chain length for the amylopectins are shown in Table 5, 5. There are no significant differences in the values of these properties for the samples. The weight-average molecular weights  $\bar{M}_w$ , for some of the samples were obtained by light scattering.

In general, the properties of the components from the berry and tuber starches are comparable; a radical change in the mode of biosynthesis of starch in the berry, compared to that in the tuber, would appear to be unlikely.

TABLE 5.5

Properties of Amylopectin Components from Starches.

Variety	Starch source	Purity (%)	$\beta$ -Limit (%)	Av. chain-length	Length of internal chain (#)	Molecular weight $\times 10^{-6}$ (1)	Radius of gyration (Å)
Dr. MacIntosh	Tuber	> 99	58	25	8	110	2280
Dr. MacIntosh	Berry	> 99	57	24	8	55	1810
Pentland Dell	Tuber	> 99	58	24	8	35	1210
Pentland Dell	Berry	> 99	58	24	8		1650
Mexican strain	Tuber	> 99	57	26	9	-	-
Mexican strain	Berry	> 99	58	26	9	-	-

\*) From results of potentiometric iodine titrations.

%) Conversion into maltose under the action of pure  $\beta$ -amylase.

\*\*) Calculated from chain-length - [(chain-length  $\times$  -limit) + 2.5].

\*) From lightscattering measurements.

SECTION 6.

Biosynthesis of Starch:

Studies on the Growth of the Starch Components.

SECTION 6. 1.

A Study of the Properties of Starches  
from the Growing Potato Tuber.

(a) Introduction.

Biosynthesis of Starch.

The state of present knowledge about the mode of biosynthesis of starch granules is the result of a somewhat slow accumulation of worthwhile facts, allied to the speculative use of these facts. However, the elucidation of the mechanism of starch biosynthesis is an extremely complex problem in which there are a large number of variables operative. The problem is both a biological and a chemical one, and to obtain a comprehensive picture of the situation, it must be considered from both of these points of view.

It is widely accepted that starch granules originate in the amyloplasts, (e.g. O'Brien, 1951) which, when examined with an electron-microscope, appear to be vesicles, "surrounded by a double membrane with few, if any, membranous structures inside." (Badenhuizen, 1963). Whistler and Thornburg (1957) in fact, used the electron-microscope to study the various stages in the formation of a granule of corn starch. These workers observed starch granules in corn endosperm as early as four days after pollination. At the earlier stages the young granules were surrounded by the amyloplast membrane, but as the granules grew they completely filled the membrane and in many cases ruptured it. Fragments of the amyloplasts were /



were seen attached to granules at all stages of maturity beyond twelve days after pollination. Despite their seemingly simple form, amyloplasts from various plants show typical structural characteristics which must have a genetical origin; the importance of the genetic influence has been clearly demonstrated in the shape and composition of starch granules from various corn varieties, (Badenhuizen, 1959).

It has been firmly established, using autoradiographic techniques, that starch granules grow by apposition i.e. deposition of successive layers. (Potato starch - Badenhuizen, 1956; bean starch - Yoshida et al, 1958). This mechanism implies structural equality of all layers of a granule, (water content excluded), and at the same time dispenses with the postulate that the granule is surrounded by a thin outer membrane. Lengthening of molecules at the periphery of the granule was thought to be the mechanism by which growth takes place. However, this theory does not take into account the inability of retrograded molecules to act as primers should synthesis be catalysed by phosphorylase. (Whelan and Bailey, 1954). Another suggestion (Macmasters et al, 1946) was that the granule was formed by a process involving rapid periodical crystallisation from a coacervate. This approach has also been employed by Badenhuizen (1963).

#### Enzymic Synthesis.

Many theories for starch biosynthesis have been postulated. One of the earliest involved the production of starch /

starch by the combined action of phosphorylase and Q-enzyme.

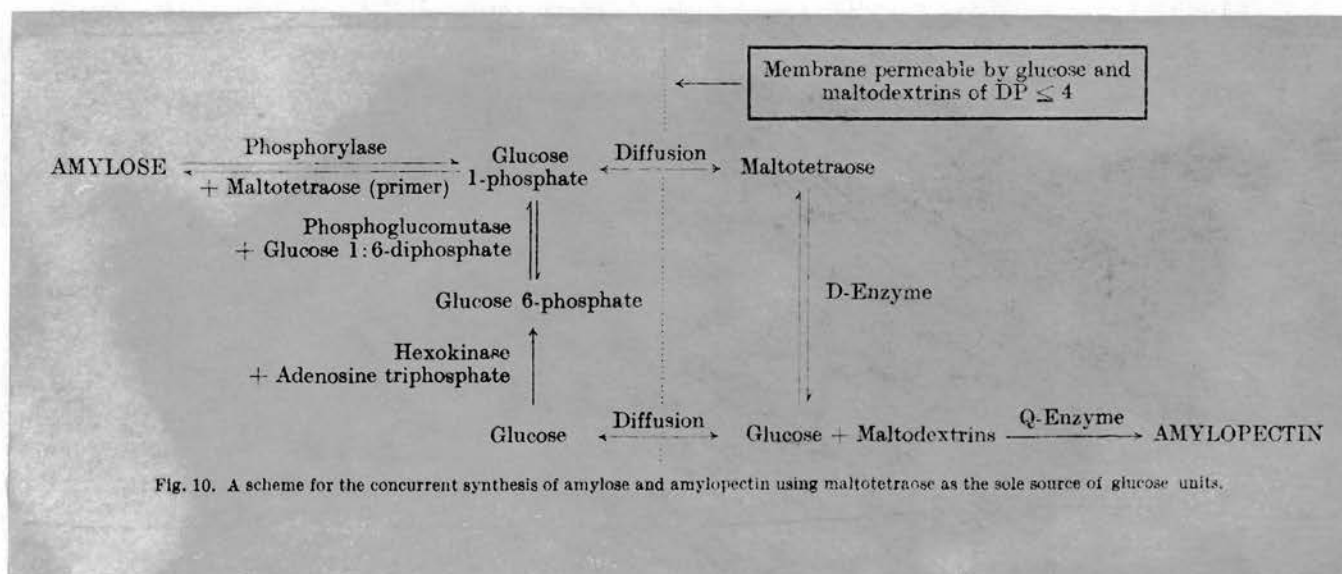
Phosphorylases, in vitro, produce linear molecules (synthetic amylose) from glucose-1-phosphate (G - 1 - P) in the presence of a primer molecule which, for potato phosphorylase, must contain at least three glucose units linked together by -1:4 glucosidic bonds. (French and Wild, 1953). The chain lengthening process takes place by addition of glucose units to the primer non-reducing end group. It is relevant to an understanding of the development of the starch granule that a high concentration of primer end-groups gives a final product which has relatively short chains, while a high concentration of G - 1 - P or a low primer concentration induces the production of long chains (Stacey, 1954).

The Q-enzymes are responsible for the production of branched molecules from the linear chains. They catalyse the conversion of some of the 1:4 links into 1:6 links and their action must be preceded by that of phosphorylase. When the substrate consists of linear molecules, the length of these chains has an influence on Q activity. Peat et al (1953) have stated that the minimum precursor chain length for rapid reaction is ca 40 glucose units. Experiments in vitro using phosphorylase and Q-enzyme have shown that only the branched component is synthesised and that the degree of branching is very largely dependent on the ratio of phosphorylase activity to the branching enzyme activity.

Considering the situation in vivo, we find that phosphorylase has been discovered to be associated with the sites /

sites of starch formation in the plastids. However, this does not necessarily mean that starch would be produced under natural conditions, as cases are known (Badenhuizen, 1955) where phosphorylase has no physiological function. While phosphorylase may be a major factor in starch synthesis, it might be logically concluded that the branching enzyme cannot be an operative factor in determining the amount of amylose present in the granule. In fact, it might not be unreasonable to suggest that amylose would have its own synthesising mechanism.

Whelan (1958) postulated the formation of amylose and amylopectin in different parts of the amyloplasts as two different processes. He supposed that within the space to be filled with synthesised starch, there may be some obstacle which prevents free diffusion of the enzymes. The obstacle, e.g. a membrane, renders possible the formulation of a hypothesis to explain how the concurrent synthesis of amylose and amylopectin could occur. This system requires two compartments separated by a semi-permeable membrane capable of passing molecules in size up to say maltotetraose (See Fig. 6.1).



In one compartment are D- and Q-enzymes and in the other hexokinase (+ adenosine triphosphate), phosphoglucomutase (+ glucose 1:6-diphosphate) and phosphorylase. The first enzyme regarded as exerting its action will be D-enzyme, which causes a disproportionation of the maltotetraose into other maltodextrins and glucose. This glucose passes through the membrane and is converted into glucose-1-phosphate (G - 1 - P) by the hexokinase - phosphoglucomutase system. Synthesis of amylose from G - 1 - P and maltotetraose will ensue and as the growing amylose chains cannot diffuse through the membrane, they cannot be attacked by the branching enzyme. Meanwhile, in the D- + Q-enzyme compartment the removal of glucose, by disturbing the equilibrium in the D-catalysed reaction, (Walker and Whelan, 1959), will lead to an increase in the average length of the maltodextrins, which in turn, will be acted upon by the Q-enzyme to form amylopectin. In the same way as the synthesised amylose will be protected from the Q-enzyme, so also will the amylopectin be unable to pass through the membrane into the phosphorylase compartment. Although G.- 1 - P and G - 6 - P will be free to penetrate into the D- + Q-enzyme compartment they will not be utilised by D-enzyme and it is not thought likely that they can act as precursors for Q-enzyme.

Many arguments have been put forward, both supporting and opposing Whelan's hypothesis. Consideration of the above scheme will show the net result to be the incorporation of three glucose units of maltotetraose into amylopectin and one /



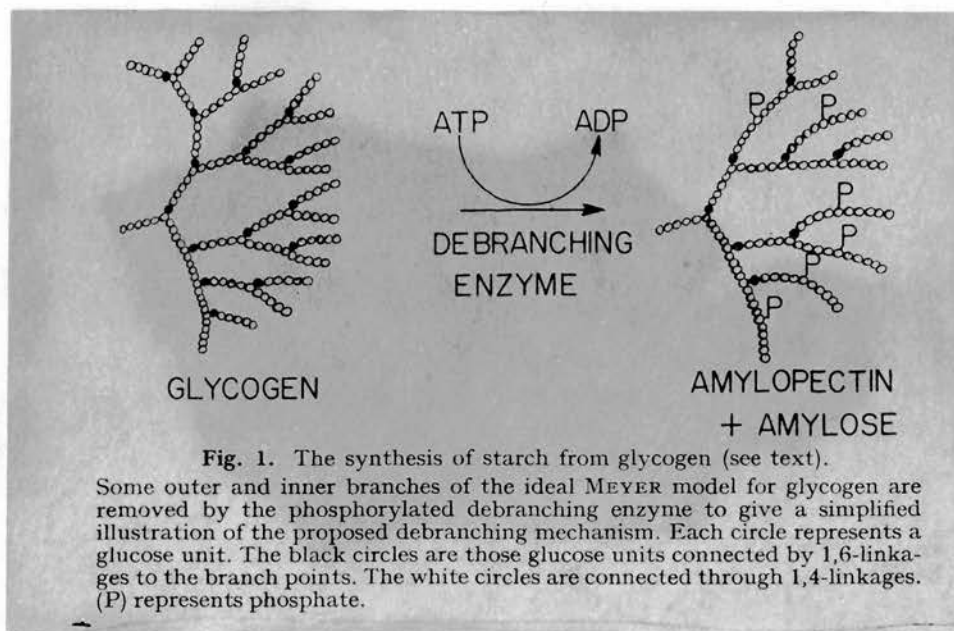
one into amylose. The resulting weight ratio corresponds to the ratio most often found in natural starches. However, since the amylose and amylopectin are produced in different parts of the amyloplast, the two components must diffuse to a common centre of the cell in order to form a starch granule. If the amylose and amylopectin molecules can migrate, there appears to be no reason why the enzymes themselves should not migrate in the same way. Another criticism (Badenhuizen, 1961) is that this mechanism does not satisfactorily explain the lack of estrified phosphorus in amylose and its presence in amylopectin.

An alternative postulate to the membrane system, (Whelan, 1958) is that the enzyme molecules become surrounded by their products of synthesis which, under favourable conditions, crystallise around the enzyme and prevent its free movement within the granule without necessarily curtailing the catalytic action of the enzyme on molecules small enough to penetrate the granule.

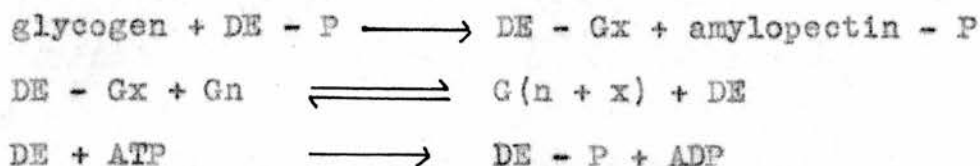
Erlander (1958) took an entirely different approach in proposing a mechanism based on phytoglycogen as an intermediate. In doing so, he supported the idea first put forward by Wolf et al (1948), who found that sweet-corn granules lie embedded within globules of glycogen. The theory requires a set of hypothetical enzymes, some of which are used to debranch glycogen to amylopectin and others to connect the liberated branches to form amylose. (Fig. 6. 2).



(Fig. 6. 2).

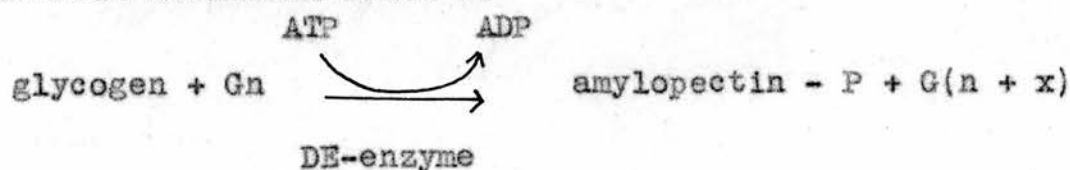


Complete scission of all the 1:6 linkages in glycogen would yield only small linear molecules; the mechanism proposed was therefore as follows:-



where DE - P represents the phosphorylated debranching enzyme, Gx the unbranched chain and Gn the receptor group. ATP and ADP represent adenosine triphosphate and adenosine diphosphate respectively.

The overall mechanism would be



The resulting linear molecules, G(n + x) can now act /

act as acceptors and the long linear chains finally formed can crystallise out of the medium before being attacked by the branching enzyme. According to Erlander, the amylopectin may also crystallise out and the variations in the rate of crystallisation cause the production of the granular rings. With regard to the above mechanism the author makes the following postulates. (1) The debranching reaction would have to be essentially irreversible. (2) The glycogen molecule may be attacked more than once by the debranching enzyme. (3) The receptor group, Gn, must have an available non-reducing glucose group and could be of any size. (4) Debranching produces longer external and possibly longer internal branches. (5) Phosphate is left at carbon atom-6 of a glucose unit in the amylopectin molecule. Phosphate may also be left on amylose, when amylose is synthesised from internal branches. The phosphate attached to the amylose or amylopectin may be removed by a phosphatase. Various objections to this theory have been put forward, not the least of which is the fact that no debranching enzyme is known which will debranch glycogen to give an amylopectin molecule. In addition there is no explanation for the presence or the action of Q-enzyme, or for the absence of glycogen in the great majority of plant sources. Thus, although this theory has useful features, it must be accepted with reservation because of its largely hypothetical nature.

The widespread discovery of the sugar nucleotides and their involvement in carbohydrate biosynthesis has led to /

to the revision of many of the older ideas. This new thinking began with a discovery by Leloir and Cardini (1957) regarding glycogen synthesis - namely, that rat-liver contains an enzyme (glycogen synthetase) which synthesises glycogen, not from  $\alpha$ -glucose 1-phosphate but from uridine diphosphate glucose (U.D.P.G.). The discovery of the nucleotide pathway in animals led to similar speculation about the synthesis of starch. It might be supposed that U.D.P.G. could be implicated in starch synthesis through a corresponding starch-synthetase, or more correctly, an amylose-synthetase, since Q-enzyme is still necessary in order to form amylopectin from amylose. The detection of such a synthetase was announced in 1960, again by Leloir and coworkers. The enzyme was found to be bound to the starch granule. The preparation reported was, in fact, a suspension of starch granules. When suspended in a solution of U.D.P.G., the glucose residues of the nucleotide were transferred to the granules, the starch molecules presumably acting as receptors. Transfer can, however, also take place to maltose, maltotriose etc., if these are included in the solution. Much more active enzyme preparations, still adsorbed on starch, were later obtained by Pottinger and Oliver (1962). These authors also observed that far more active preparations are obtained if the granules are isolated in 0.05 M sucrose-citrate medium, indicating that the starch synthetase can be separated from the granules.

Further research into the sugar nucleotides produced one which reacted even more quickly with amylose-synthetase, namely /

namely adenosine diphosphate glucose, (A.D.P.G.) and Recondo and Leloir (1961), found a plant enzyme that synthesised A.D.P.G. from substances known to occur naturally i.e. adenosine triphosphate and glucose 1-phosphate.

An important contribution to the problem of co-existence of two very widely differing polysaccharides and their synthesis in close proximity, was made by Nelson and Rines (1961). These workers compared normal-maize and waxy-maize starch for their contents of amylose-synthetase. While normal-maize contained the enzyme, the latter contained no enzyme synthesising starch from U.D.P.G. The conclusion reached was that amylose synthesis and amylopectin synthesis are not closely linked and that the glucose units of each polymer are derived from different precursors. Further evidence of this nature is furnished by Leloir's report on the relative labelling of amylose and amylopectin when the amylose-synthetase preparation (starch suspension) was incubated with  $^{14}\text{C}$ -labelled U.D.P.G. A preference for the labelling of amylose was shown.

From the foregoing facts, it may be concluded (Whelan, 1963), that amylopectin is synthesised by phosphorylase and Q-enzyme from  $\alpha$ -glucose 1-phosphate and that amylose is formed from one of the sugar nucleotides, probably A.D.P.G., by synthetase. (Figure 6. 3) (See following page). It should, however, be pointed out that A.D.P.G. has not yet been detected in plants.

Figure 6. 3.

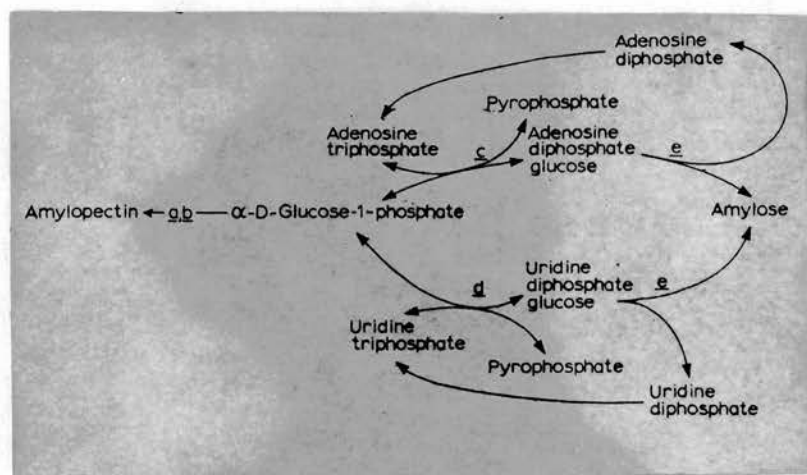


Fig. 3. Biosynthesis of amylopectin and amylose  
(Key: *a* phosphorylase; *b* Q-enzyme; *c* adenosine triphosphate: α-D-glucose 1-phosphate adenylyl transferase; *d* uridine triphosphate: α-D-glucose 1-phosphate uridylyl transferase; *e* amylose synthetase).

The conclusions of Badenhuisen (1963) are somewhat more cautious. "Our conclusions must be that phosphorylase is the predominant starch synthesising enzyme and that it can be excellently coordinated with a U.D.P.G. or related system."

These current findings are not inconsistent with the idea that amylopectin is the basic structural element of the starch granule and when amylose is present, it is deposited within the amylopectin framework. Also, the discovery of separate pathways to amylose and amylopectin makes it easier to envisage why amylose continues to exist in the presence of /



of Q-enzyme.

A detailed study of changes in the structure of amylose and amylopectin with increasing maturity of a plant would provide another angle of approach to the aforementioned problems; the data produced should aid in evaluating the various current theories of starch biosynthesis.

(a) Introduction (Continued).

Studies on Changes in the Properties of Starch with Plant Maturity.

It is now well established that there are changes in the amount of starch and in the characteristics of the granules stored in the growing plant. Such studies have been carried out on maize (Wolf, MacMasters, Hubbard, and Rist, (1948); Erlander, 1960), sweet corn (Maywald, Christensen and Schoch, 1955), barley (Harris and Macwilliam, 1958), smooth and wrinkle-seeded peas (Greenwood and Thomson, 1962), tobacco leaves (Matheson and Wheatley, 1962 and 1963) and potato tubers (Stepanenko and Afanasyeva, 1957; Thomson, 1961).

The general pattern emerging from these investigations is, that as the plant matures, the starch content increases whilst there is an increase in both the average size of the granule and the amount of the amylose component that it contains. In the particular case of potato starch, the work of Halsall, Hirst, Jones and Sansome, (1948) indicated that the percentage of amylose remained constant for two varieties of the growing tuber. There is evidence, however, that the amylose content may vary with the botanical variety of tuber (Anderson and Greenwood, 1955) while results on the starch from the tuber and shoots of the sprouting tuber (Banks and Greenwood, 1959) indicated that immature potato starch may contain less amylose than mature granules.

In a study on the structure of amylopectins and amyloses isolated from potato tubers during their maturation under /

under different conditions of cultivation, Stepanenko and Afanasyeva (1957) came to several interesting conclusions regarding variations in the fine structure of the components with tuber development. Regardless of the agrobackground (i.e. extent of fertilisation of the soil) the molecular weight of amylose was found to increase as the tuber matured. The molecular weight of amylopectin, however, increased under low agrobackground conditions and was accompanied by a very small increase in the length of unit chain; but, with high agrobackground, a decrease in the molecular weight with a decrease in unit chain was observed. In general, it was concluded that in all cases, increase in molecular weight was associated with an increase in the length of unit chain and vice versa. The data were interpreted on the basis of biosynthesis of starch by phosphorylase and a branching enzyme - Q-enzyme. The activity of the branching enzyme was considered to "lag behind" that of the phosphorylase as the polysaccharide molecules increased in size. The above authors, however, made no attempt to relate fine structure variations to granule size or indeed to a specific tuber size and while their results indicate the existence of such variations, there is also an underlying need for more precise control of the tuber samples and a more rigid definition of tuber maturity.

Thomson (1961) isolated starch from tubers of varying sizes and his findings were in general agreement with conclusions drawn by Stepanenko and Afanasyeva for tubers from a low agrobackground.

Apart /

Apart from these conclusions and the work on pea starches (Greenwood and Thomson, 1962) the evidence in the literature regarding changes in the fine structure of the components during growth is sparse. This work, therefore, examines more closely, the physical and chemical properties of starch isolated from potato tubers at various stages of growth. In addition, the properties of the amylose and amylopectin components have been studied.

Although the presence of two components in starch is generally accepted, the results of various workers point to the existence of a third component with properties different from those of amylose and amylopectin. As a result of iodine affinity studies of amylose and amylose subfractions, Lansky et al (1949) suggested that there was present in maize starch 5 - 7% of a third component. This polysaccharide was precipitated by "Pentanol" but not by n-butanol. By comparison of the observed  $\beta$ - and  $\beta + \alpha$ -amylolysis limits for potato starch and artificial mixtures of amylose and amylopectin, Peat, Pirt and Whelan (1952) concluded that the composition of starch could not be accurately expressed in terms of two components. These workers held the view that there is present in starch a third component having a slightly higher blue value but a lower  $\beta$ -amylolysis limit than amylopectin.

Cowie and Greenwood (1957), in a critical study of fractionation procedures, found that the initial amylose-thymol complex /

complex was only ca. 75% pure. The contaminant, termed "thymol-amylopectin" and isolated from the supernatant liquid after recrystallisation of the thymol-amylose complex with n-butanol, was again found to have a higher iodine affinity than amylopectin.

An anomalous branched fraction was isolated from wheat starch by Perlin (1958). This component (Amylopectin C), comprising 5 - 10% of the total starch, had a  $\beta$ -amylolysis limit some 7% lower than that of the normal amylopectin; a minor component has also been found in waxy maize starch, (Erlander and French, 1958). The behaviour of this component in solution was found to be typical of a glycogen-type polysaccharide and it was proposed that the material was, in fact, glycogen. These authors further suggested that the thymol-amylopectin of Cowie and Greenwood (1957) was also glycogen. In 1959, Banks and Greenwood further examined the anomalous thymol-amylopectin and also isolated the corresponding component from rubber-seed starch. The product was again found to have a significantly lower average chain length and  $\beta$  - amylolysis limit than amylopectin.

Finally, the intermediary fractions from high-amylose corn starch have been characterised by Whistler and Doane (1961) and the products found to indicate a degree of branching and a shape intermediate between amylose and amylopectin.

In view of the indeterminate nature of this third component and the importance with regard to biosynthetic theories, the thymol-amylopectins were also isolated and examined.



(b) Experimental.

Differences in the sizes of tubers on any one plant have been attributed to unequal rates of growth rather than to differences in the age of the tubers, (Clark, 1921); the autoradiographic researches of Badenhuisen and Dutton, (1956) appear to confirm this conclusion. On the basis of these statements, it would have been impossible to make a choice of samples dependent on the absolute age of the individual tuber. Likewise, a gradation dependent on times of harvesting would have been unreliable. Recourse was had, therefore, to measurement of tuber size as a criterion of maturity.

The potatoes, (variety Pentland Crown) were grown at the Scottish Plant Breeding Station, Pentlandfield, Roslin, Midlothian in the 1962 season and were supplied through the courtesy of Dr. W. Black. Tubers were harvested at intervals of ten days after the appearance of leaves on the plant and separated into sizes varying from less than 1 cm. to 15 cm. in longitudinal diameter.

Starch was isolated from the different sized tubers and was purified by the method given earlier (Section 2). In a similar manner, starch was also isolated from the shoots, (i.e. the underground stems devoid of tubers) of mature plants and from the original seed-tuber. The rather unusual preservation of the seed-tuber in an intact, non-rotted /

non-rotted state was a consequence of the particularly dry growing season.

The method used for estimation of the percentage of starch in the plant material is detailed in Section 2.

### Characterisation of the Granular Starches.

#### Estimation of Granular Size.

A measure of granular size was obtained by enlarging photomicrographs (four to five fields per sample) to give a final magnification of about 500 diameters and by measuring with a rule (to the nearest millimetre) the size of at least 500 granules of each starch. In view of the ellipsoidal nature of potato starch granules, each was characterised by the length of its major axis, (d).

Number average particle diameters were calculated from

$$\frac{\sum n_i d_i}{\sum n_i} \quad \text{where } n_i \text{ is the number of granules of diameter } d_i.$$

The following estimations were carried out according to the methods detailed in Section 2.

- (a) Gelatinisation temperatures.
- (b) Percentage amylose.
- (c) Percentage nitrogen.
- (d) Percentage phosphorus.

#### Fractionation of the starches.

The starch samples were treated with liquid ammonia prior to their dispersion in water, by boiling for 1 hour in a nitrogen atmosphere. Thymol was added to the dispersions /

dispersions to precipitate the amyloses which were subsequently purified by recrystallisation (twice) as the n-butanol complexes. The amylopectin was obtained by freeze drying the supernatant liquors after removal of the amylose complex. In each fractionation, the anomalous amylopectin fraction described by Banks and Greenwood, (1959) was isolated from the supernatant liquor after the first recrystallisation of the amylose-thymol complex with n-butanol.

Characterisation of the fractionation products.

The amylose fractions were characterised with respect to iodine affinity,  $\beta$ - and  $\beta + Z$ -amylolysis limits and limiting viscosity number in M potassium hydroxide at 25° C. In addition the phosphorus content of a few samples was determined. Likewise sedimentation coefficients were determined for some of the amyloses and the resultant figures substituted in the Scheraga - Mandelkern equation to give approximate molecular-weights.

The properties determined to characterise the amylopectins were:- nitrogen content, phosphorus content, iodine affinity, -amylolysis limit, chain length and, for a few samples, the sedimentation coefficients in 0.1 M sodium chloride at 20°C. The weight-average molecular weights of some of the amylopectins were determined by light-scattering, in aqueous solution, using a Brice-Phoenix light-scattering photometer (Model 1000D) as described by Greenwood and Thomson, (1962). The latter measurements were kindly carried out by Mr. R. Geddes.

The /

The "thymol-amylopectins" were all characterised with respect to iodine affinity whilst  $\beta$ -amylolysis limits and apparent chain lengths were determined for some of the samples. Likewise, sedimentation coefficients in 0.1 M sodium chloride were obtained using a Spinco, Model E, ultracentrifuge.

Subfractionation of the "thymol-amylopectin" was attempted as follows.

Ethanol was slowly added, with stirring, to a solution (0.25%; 100 ml; sample 5) of the polysaccharide in dimethyl sulphoxide at 35°C, till, after about 25% by volume of ethanol had been added, a permanent turbidity was observed. Centrifugation at 17,000 r.p.m. gave a gel-like product which was thoroughly washed with ethanol, redissolved in oxygen-free water and filtered. The polysaccharide was recovered by freeze drying the solution. Subsequent stepwise additions of ethanol gave further subfractions, the last few of which tended to be amorphous rather than gelatinous. In all, seven subfractions were obtained.

(c) Results and Discussion.

Properties of the Granules.

The properties of the starch granules are shown in Table 6. 1. It may be seen that, as the tuber increases in size, the starch content, the number-average granule diameter, the iodine affinity and, hence, the percentage amylose also increases. These trends, illustrated in Fig. 6. 4, are in agreement with the general ideas referred to in the introduction.

Whilst it has been established that the starch content of both corn, (Wolf et al, 1948) and sweet-corn kernels (Maywald et al, 1955) increases with maturity, this is the first quantitative measurement of the overall trend for potato tubers. It may be seen, (Fig. 6. 4a), that the starch content tends to a limiting value. The results for corn starch are similar. (c.f. Wolf et al, 1948).

Similarly, the fact that large granules gelatinise more easily than small ones is well known. Fig. 6. 4b. shows that as the tuber size increases, the gelatinisation temperature appears to be falling to a limiting value. The reverse tendency may be observed in the plot of percentage amylose against tuber size. This curve also tends to a limiting value and, in fact, on graphing gelatinisation temperature against percentage amylose, an inverse straight-line relation is obtained, (Fig. 6. 4c). Since amylopectin is the basic structural material of the granule, (Badenhuizen, N.P., 1963 ) it might not be unreasonable to expect that, as the amount of amylopectin present in the granule decreases /



TABLE 6. 1.

## Properties of the Starch Granules.

Sample No.	Tuber size (cm)	Starch Content (%)	No. Average granular diameter ( $\mu$ )	Average gelatinization temperature (°C)	Iodine Affinity	Amvlose (%)#	Phosphorus (%)	Nitrogen (%)
1	0-1	5.0	10.5	70.0	2.45	12.5	0.05	0.003
2	1-2	5.6	N.D.	69.5	2.56	13.2	0.05	0.01
3	2-3	6.4	20.	68.5	2.70	13.9	0.03	0.012
4	3-4	8.3	N.D.	66.5	2.85	14.6	0.03	0.007
5	4-5	9.2	25	65	3.20	16.4	0.04	0.008
6	5-6	11.0	28	63.5	3.35	17.2	0.05	0.007
7	6-7	13.4	32	63.5	3.55	18.2	0.04	0.010
8	7-8	17.0	N.D.	62.5	3.60	18.5	0.05	0.011
9	8-9	17.5	45	61.5	3.70	19.0	0.04	0.008
10	9-10	17.5	N.D.	61.5	3.74	19.2	0.04	0.006
11	10-11	18.0	45	62.0	3.85	19.8	0.06	0.01
12	14-15	N.D.	N.D.	60.5	3.90	20.0	0.04	0.007
13	15-16	N.D.	N.D.	59.5	3.90	20.0	0.04	0.006

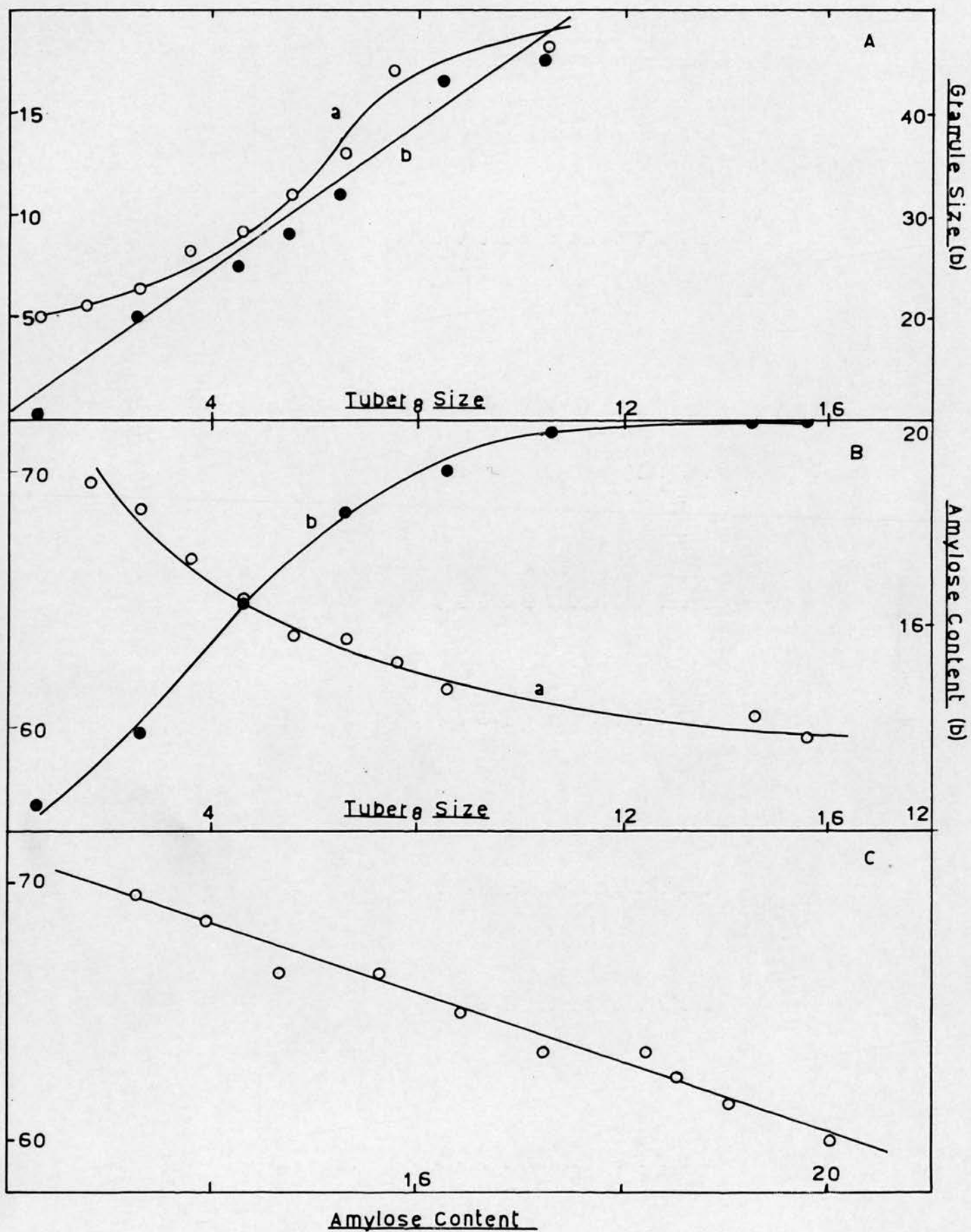
14	Residual Seed Tuber	3.0	30	61.5	3.80	19.5	N.D.	0.05
15	Shoot	5.0	16	70	2.40	12.4	N.D.	N.D.

\* Expressed as mg. of I<sub>2</sub> bound/100mg. of starch.

# Calculated from iodine affinity.

N.D. Not determined.

FIG. 6.4.



decreases, so any alterations in granular architecture would be reflected in changes in the temperature at which the structure breaks down, namely the gelatinisation temperature.

The relation between tuber size and number-average granule diameter is not well defined, (Fig. 6. 4a); the experimental points could be joined either by a sinusoidal-type curve, or within experimental error, a straight line. While granule size might not be expected to increase indefinitely, nevertheless, if attention is restricted to the limits of actual experimental observation, the straight line plot is the better interpretation of the points shown. Thus a direct relation between tuber size and granular diameter might be reasonably deduced. (c.f. Wolf et al, 1948).

It will be noted (Table 6. 1) that the granules from the shoots, (sample 15) are larger than those from the smallest size of tuber, (sample 1), but it must be reiterated (see Experimental) and emphasised, that the shoots were obtained at a later stage of plant development than were the small tubers. The smallest tubers, (sample 1), were in reality, nothing more than nodules on the end of the shoots and, being extremely small in size, were collected at various times in order to provide sufficient starch for fractionation to be carried out. The first sample which could be called a definite tuber, was sample 2.

The variation in the percentage of nitrogen present in the granules is purely random and merely reflects the degree /

degree of purity with respect to contaminating protein, which varies from 0.019% to 0.075% in the 'normal' tubers. The high value of 0.05% nitrogen, (0.30% protein) in the residual seed-tuber is presumably due to the difficulty of separating the small amount of starch present from the rest of the tuber material. This unusual sample was of a different, somewhat more fibrous, consistency from all the other tuber samples.

The percentage of phosphorus also varies randomly. This finding is at variance with previous workers, some of whom have found the percentage of phosphorus to be greater for larger granules, (Samotus and Schwimmer, 1962; Thomson, 1961), others of whom report that small granules contain more phosphorus, (Higginbotham and Richardson, 1940; Walger et al, 1962). In this work the variation in the amount of phosphorus present may be interpreted as due to the presence of varying amounts of compounds, other than starch, containing phosphorus e.g. nucleic acids, phosphoproteins and phospholipids. Bice et al (1945) stated that the phosphorus content of starch from wheat kernels was not found to change materially with maturity.

#### Properties of the Amyloses.

The properties of the amylose components are shown in Table 6. 2). The amyloses obtained were all of very high purity, as shown both by the results from iodine affinities and the complete conversion into maltose by the combined action of  $\beta$  - and Z-enzymes. The  $\beta$  -amylolysis limits show a distinct trend - decreasing from a maximum value of 92% /

92% for sample 1 to a lower limit of 81% for samples 12 and 13. At the same time, the limiting viscosity number shows a marked increase with increase in tuber size. The graph of  $\beta$ -limit and viscosity against tuber size (Fig. 6.5a) shows a tendency for both these characteristics to reach a limiting value. However, graphing the same properties against the percentage amylose (Fig. 6. 5b) demonstrates that viscosity is directly related to the amylose content of the granule while the  $\beta$ -limit is inversely related. The correlation between  $\beta$ -limit and viscosity is illustrated in Fig. 6. 5c.

The trend in  $\beta$ -amylolysis limits offers a sound indication that the barrier to complete hydrolysis by pure  $\beta$ -amylase is a natural one and not an artificial product of the fractionation procedure. If the barrier were an experimental artifact it would be introduced into the amyloses in equal quantities, since they all undergo the same fractionation procedure. Since, however, the amount of barrier varies with the length of the amylose molecule, it is probably introduced into the amylose molecule during the growth of the plant. Also, it may be that the amylose, being polydisperse, contains a small amount of a polysaccharide having a few branch points. Increasing the amylose content of the granule would lead to the presence of larger amounts of a branched subfraction and hence to the observed decrease in  $\beta$ -amylolysis limit.

The limiting viscosity number for the shoot amylose is larger than that for the smallest tuber size (sample 1), which /



FIG. 6.5.

- 138a-

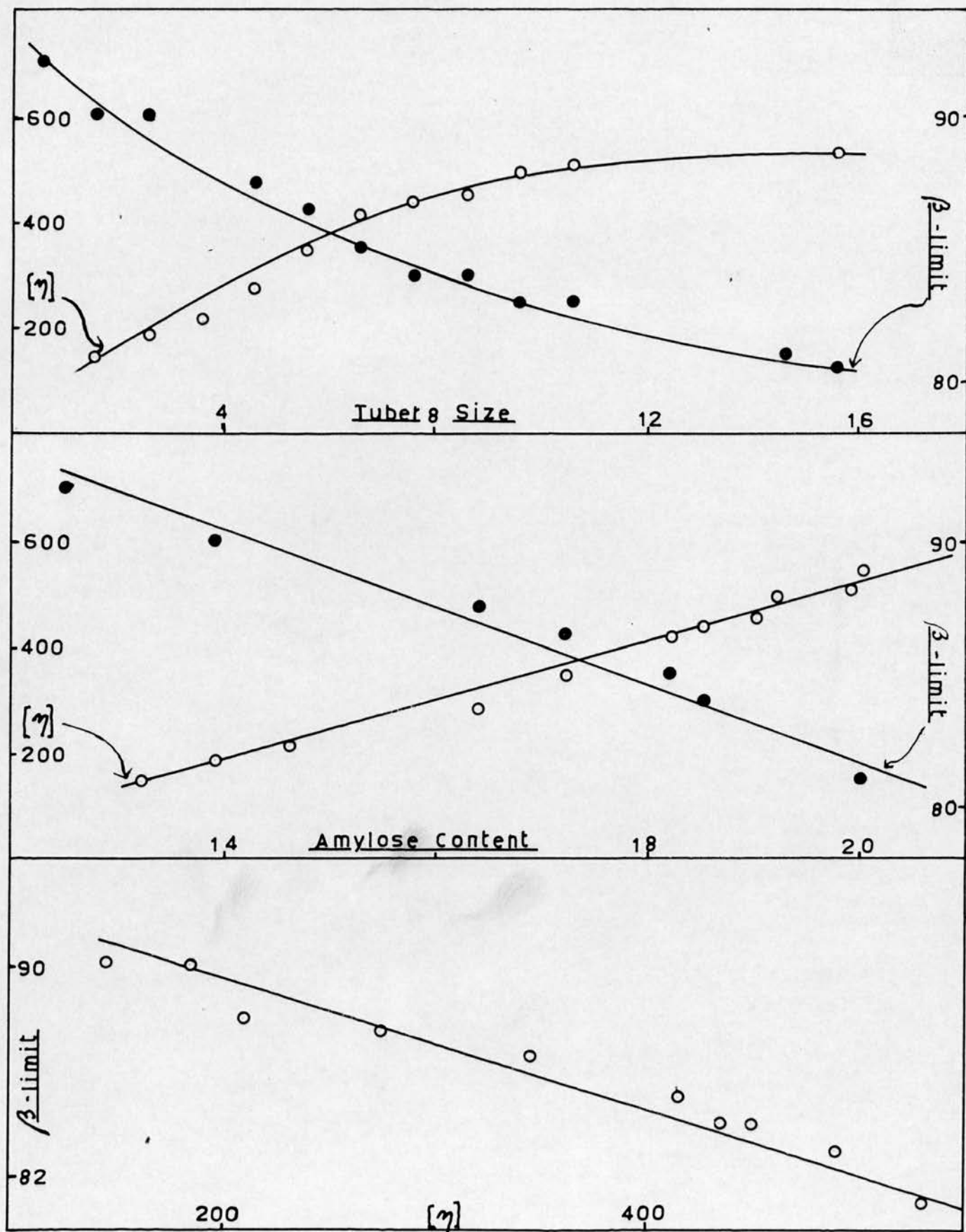


TABLE 6. 2.

Properties of the Amyloses from the Starches.

Sample No.	Tuber Size (cm)	Purity (%) <sup>*</sup>	$\beta$ -limit <sup>†</sup> (i)	$\beta$ -limit <sup>†</sup> (ii)	$[\eta]$ X	Degree of Polymerisation <sup>xx</sup>
1	0-1	98	92	101	305 <sup>""</sup>	2200
2	1-2	98	90	100	145	1100
3	2-3	98	90	99	185	1400
4	3-4	98	88	100	210	1600
5	4-5	98	88	101	276	2100
6	5-6	98	87	101	345	2600
7	6-7	98	85	101	415	3100
8	7-8	98	84	100	435	3200
9	8-9	98	84	100	450	3300
10	9-10	98	83	102	490	3600
11	10-11	98	83	101	505	3700
12	14-15	98	81	99	540	4000
13	15-16	98	81	99	530	4000

14	Residual Seed Tuber	98	86	100	310	3300
15	Shoot	98	89	100	390	2900

\* Calculated from iodine affinities.

† Percentage conversion into maltose on treatment with (i)  $\beta$ -amylase (ii)  $\beta$ -amylase and Z-enzyme.

X Determined in MKOH at 25°C.

"" Average value from two independent fractionations when  $[\eta] = 300$  and 310 respectively.

xx Calculated from D.P. =  $7.4 \times [\eta]$ .

TABLE 6. 3.

Sample	Phosphorus %	$[\eta]$ <sup>*</sup>	$S_0 \times 10^{13}$ <sup>†</sup>	Molecular Weight $\times 10^{-6}$ <sup>++</sup>
3	N.D.	210	4.1	0.2
5	0.002	N.D.	-	-
7	0.002	490	7.1	0.8
10	0.002	N.D.	-	-
11	N.D.	600	9.0	1.2
12	0.003	N.D.	-	-
13	N.D.	630	9.5	1.3

\* in 0.15 M potassium hydroxide at 20°C.

N.D. Not determined.

† Sedimentation coefficients determined in 0.15 M potassium hydroxide.

++ Calculated from the relationship of Scheraga and Mandelkern

which, in turn, is larger than that for sample 2. The unexpected large viscosity for the shoot amylose may be explained by the fact that the shoots were taken from more mature plants, as explained earlier. Approximate number average degrees of polymerisation were calculated from the relation  $D.P. = 7.4 \times [\eta]$  (Cowie and Greenwood, 1957). While these values are not thought to be precise, they are of the correct order of magnitude and provide a useful measure of relative size. An increase in the molecular size of the amylose with increasing maturity is indicated.

Another approach to this problem was to determine the sedimentation coefficient for the amylose samples and, hence, weight-average molecular weights could be derived from the equation of Scheraga and Mandelkern (1953). Although the resultant molecular weights cannot be considered to be exact since, strictly speaking, the relation holds only for linear polymer, the results (Table 6.3) confirm the above trend, i.e. the molecular weight of the amylose increases as the tuber matures. These findings on molecular weight and viscosity are in agreement with the observations of Stepanenko and Afanasyeva (1957) for potato amylose; the trends in  $\beta$ -limit and viscosity are also in accordance with previous results for pea amyloses (Greenwood and Thomson, 1962) and potato amylose (Thomson, 1961).

Values for the phosphorus content of some of the amyloses are also shown in Table 6. 3. The values found are extremely small, but it is felt that they are significant.

Minute /

Minute amounts of phosphorus could be accounted for by the presence of a small percentage of contaminating amylopectin. However, while it is extremely unlikely that amylopectin is present, it is possible that a minor portion or subfraction of the amylose could contain, not only branch points as suggested above, but also bound phosphorus. Though significant amounts of phosphorus are present, the values are too small for any valid comment to be made on the way in which the phosphorus content changes with increasing amylose chain-length.

#### Properties of the Amylopectins.

The properties of the amylopectins, obtained from the starches, are shown in Table 6, 4. As for the starches, both the nitrogen and phosphorus contents vary randomly. The nitrogen values are larger than would be expected from the protein content of the starches; this may be due to the formation of amino groups in the amylopectin and not to the presence of unduly large amounts of protein.

The low iodine-affinities of the amylopectins denote a high degree of purity with respect to contaminating amylose; the  $\beta$ -amylolysis limits may also reflect the purity since they are slightly lower than most literature values. (Greenwood, 1956).

The  $\beta$ -amylolysis limits decrease with increasing tuber size; the chain lengths, also, show a decrease with maturity, while there is a rise in the weight-average molecular weight. (Fig. 6.6a). These conclusions are somewhat at /

TABLE 6. 4.

## Properties of the Amylopectins from the Starches.

Sample No.	Tuber size (cm)	Nitrogen %	Phosphorus %	Iodine # Affinity	Purity (%)	$\beta$ -limit xx	Chain Length	Internal Chain Length	Molecular Weights x 10-6
1	0-1	0.027	0.062	0.12	99.4	56	26	9	9
2	1-2	0.028	0.052	0.02	99.9	56	26	9	N.D.
3	2-3	0.031	0.055	0.02	99.9	55	26	9	N.D.
4	3-4	0.029	0.050	0.04	99.8	54	24	9	23
5	4-5	0.036	0.050	0.03	99.8	54	23	8	N.D.
6	5-6	0.022	0.061	0.06	99.7	53	23	8	N.D.
7	6-7	0.033	0.048	0.20	99.0	52	23	9	35
8	7-8	0.035	0.053	0.04	99.8	52	24	9	N.D.
9	8-9	0.033	0.059	0.02	99.9	51	22	8	N.D.
10	9-10	0.031	0.050	0.04	99.8	51	22	8	37
11	10-11	0.042	0.062	0.25	98.7	52	22	8	N.D.
12	14-15	0.022	0.050	0.15	99.3	51	21	8	60
13	15-16	0.034	0.048	0.05	99.7	51	21	8	130

14	Residual Seed Tuber	0.020	0.05	0.04	99.8	53	24	9	N.D.
15	Shoot	0.045	0.04	0.03	99.8	57	33	12	N.D.

# Expressed as mg. I<sub>2</sub> bound/100mg starch

/ Calculated from iodine affinities

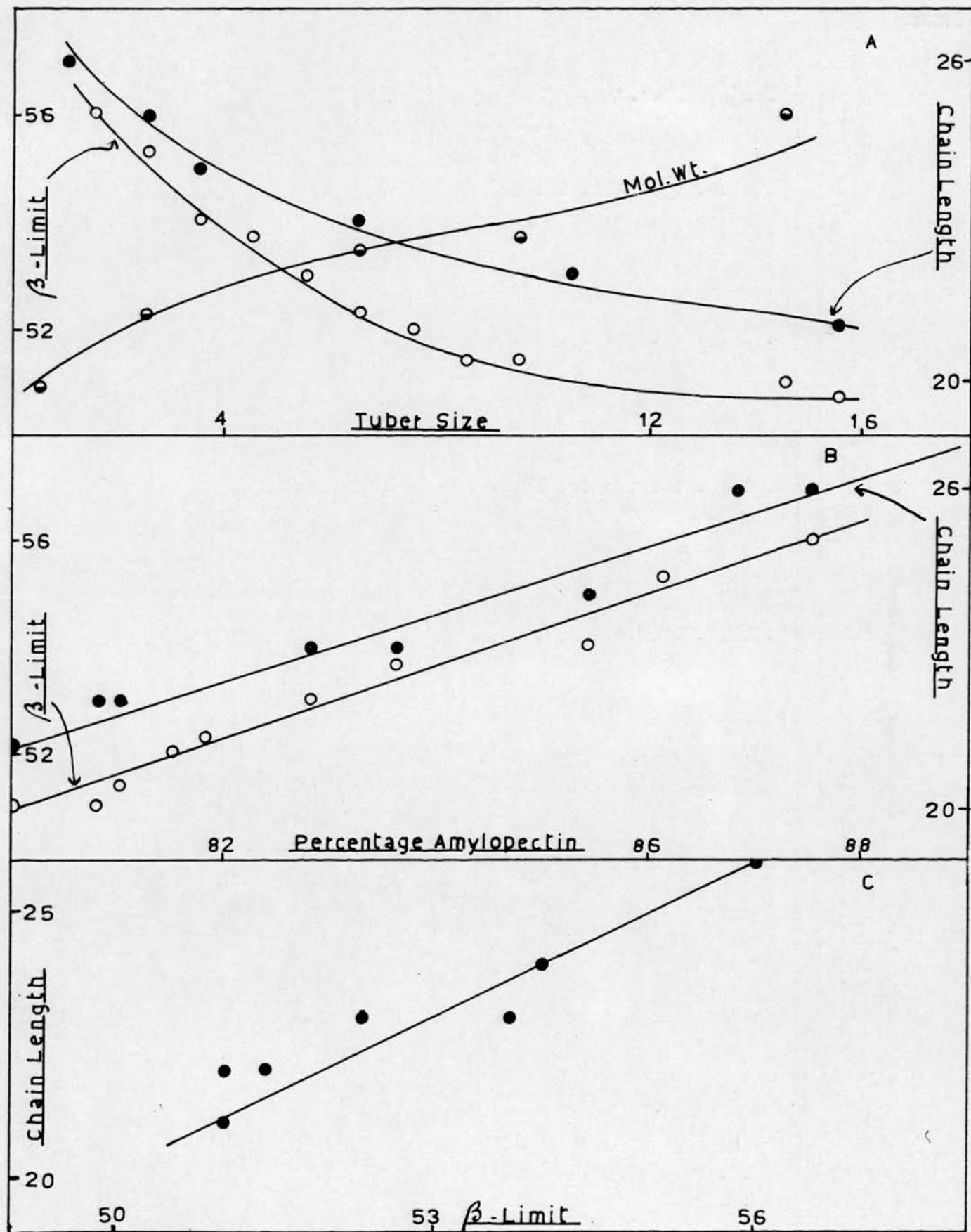
xx Percentage conversion into maltose on treatment with  $\beta$ -amylase/ Calculated from { chain length - (chain length x  $\beta$ -limit) + 2.5 }

// Weight average molecular weights obtained from light-scattering measurements.

N.D. Not Determined.



FIG. 6.6.



at variance with earlier reports in the literature; for instance, both, for pea amylopectins (Greenwood and Thomson, 1962) and for potato amylopectin (Thomson, 1961) the process of maturation has been found to be associated with an increase in chain-length and in  $\beta$ -amylolysis limit. However, no molecular weights were quoted by these authors. Stepanenko and Afanasyeva (1957), on the other hand found that, if potatoes were grown under poor conditions of fertilisation, the chain-length of the amylopectin was reduced as the tuber developed. Since the molecular weights quoted were obtained by a chemical method (Meyer et al, 1949) rather than by a more sensitive method such as lightscattering, the observed decrease in molecular weight with maturity must be accepted with reservation. It may be relevant to note that, although the potatoes used in the present study were grown on a well fertilised soil, the growing season (1962) was exceptionally dry. The nutrient salts, which are normally distributed in the upper layers of the soil, would not, therefore, have been as readily available to the plant as they would have been in more normal conditions. Thus a parallel may conceivably be drawn with the 'low agro-background' conditions of Stepanenko and Afanasyeva (1957).

It may be seen from Fig. 6. 6b that the chain-length and  $\beta$ -amylolysis limit increase with increase in the amylopectin content of the granule and that, within experimental error, the relations are linear. Fig. 6. 6c shows the graph of chain-length against  $\beta$ -amylolysis limit.

The /

The correlation between maturity of the granule, average length of unit chain and  $\beta$ -amylolysis limit may be more simply expressed as an increase in the degree of molecular branching as the granule develops. The increased branching would appear to be related to the external chains, since the calculated internal chain-length remains constant. Here a parallel may be drawn with the properties of the amylose components. These also exhibit a reduction in  $\beta$ -amylolysis limit, which might be associated with an increase in molecular branching.

The amylopectin from the shoots (sample 15) is of unusually long external and internal chain length. (c.f. Banks and Greenwood, 1959). This cannot be caused by contaminating amylose since the amount of iodine bound is small.

Ultracentrifugal examination of some amylopectins in 0.1 M sodium chloride solution provided values for the sedimentation coefficients of samples 1, 5, 10 and 13 of 200, 250, 350 and 530  $\times 10^{-13}$  respectively. These figures lend weight to the argument that the molecular weight of the amylopectin increases as the tuber matures.

#### Properties of the Thymol-Amylopectins.

The properties of the thymol-amylopectin fractions are shown in Table 6. 5. The iodine affinity is higher than for amylopectin and this may, with justification, be attributed to the presence of contaminating amylose. The amount of polysaccharide obtained (1 - 5%) is lower than values quoted in the literature, but it must be noted that different /

TABLE 6. 5.

Properties of the "thymol-amylopectins".

Sample No.	Tuber Size (cm)	Yield (% of Starch)	Iodine Affinity	Contaminating Amylose (%)	Sample No.	$\beta$ -limit	Chain Length
1	0-1	2.4	0.44	2.3	1	50	26
2	1-2	0.8	0.25	1.3	3	50	N.D.
3	2-3	2.6	0.38	1.9	5	52	19
4	3-4	0.7	0.42	2.2	7	52	13
5	4-5	4.6	0.40	2.1	9	52	N.D.
6	5-6	2.2	0.42	2.2	11	54	12
7	6-7	2.4	0.25	1.3	13	53	N.D.
8	7-8	1.5	0.22	1.1	15	50	N.D.
9	8-9	2.0	0.25	1.3			
10	9-10	3.0	0.40	2.1			
11	10-11	2.8	0.45	2.3			
12	14-15	2.0	0.13	0.7			
13	15-16	2.5	0.65	3.4			
14	Residual Seed Tuber	3.1	1.25	6.4			
15	Shoot	1.4	0.30	1.5			

N.D. = Not determined.

\* Expressed as  $\text{mgI}_2$  bound/100mg. starch  
 / Calculated from iodine affinities.

TABLE 6. 6.

Sedimentation of thymol-amylopectin.

Sample	Sedimentation Coefficient $\times 10^{13}$			
2	Homogeneous	3.0		
6	Homogeneous	11.0		
9	Heterogeneous	Fast moving component	130	
		Slow " "	11.0	
13	Heterogeneous	Fast " "	215	
		Slow " "	19.0	

different methods of isolation have been used. Indeed, it is questionable whether the various methods used result in identical subfractions of the original starch granule.

The  $\beta$ -amylolysis limits are lower than the corresponding values for the amylopectin samples, even without correction for the contaminating amylose. NO trend is apparent. The average lengths of unit-chain show a rapid decrease with increasing tuber size. The values obtained are lower than those for the amylopectins, but estimation was difficult, due to an excessive amount of overoxidation. This, in itself, would point to the presence of amylose, or, at any rate, of easily oxidisable reducing end-groups.

Examination of some samples (0.5% conc. in 0.1 M sodium chloride) in an ultracentrifuge led to the results shown in Table 6.6. While samples 2 and 6 were apparently homogeneous, samples 9 and 13 contained two components. The sedimentation coefficients of the fast-moving components are of the same order of magnitude as those of the amylopectins; the slow moving components correspond in molecular size, to amyloses. Both the slow and fast moving components show an increase in sedimentation coefficient with increase in tuber size. However, the ultracentrifugation of mixtures of two components is complex (Johnston and Ogston, 1946); in a mixture of fast and slow moving materials the apparent concentration of the former is drastically reduced. Unpublished observations in this laboratory, carried out by Mr. B. Calloway, have shown that this effect is very pronounced /



pronounced when mixtures of amylose and amylopectin are studied. Therefore, it is not possible to obtain a quantitative estimate of the amounts of the two components in samples 9 and 13 and, indeed, it is thought that the apparent homogeneity of samples 2 and 6 may be explained on the basis of a Johnston-Ogston boundary anomaly.

Further evidence of the presence of amylose was obtained by ultracentrifugal examination of a solution of sample 14, (0.3% conc. in 0.1 M sodium chloride). A partition cell was used to separate the slow moving component. On staining a portion of the solution of the slow moving component with a solution of iodine (0.2%) in potassium iodide (2%), a blue colour was formed. The iodine-polysaccharide complex had a wavelength of maximum absorption of 595 - 600  $m\mu$ . This corresponds to the blue colour formed by the amylose-iodine complex. The parent polysaccharide gave a blue colour ( $\lambda$  max. 580  $m\mu$ ), when stained with a small amount of iodine, but a red to violet colour ( $\lambda$  max. 560  $m\mu$ ) in the presence of excess iodine.

Separation was also attempted using a Sephadex (G25) column and an aqueous solution of sample 14, (20 mg. in 10ml.). The first fractions to be eluted (i.e. those of smallest molecular size) gave a blue colour ( $\lambda$  max. 600  $m\mu$ ) on staining with iodine, while later fractions gave a red colour. ( $\lambda$  max. 560  $m\mu$ ). Only partial separation of intermediate fractions was obtained and the samples were not sufficiently large for isolation and further characterisation. However, the /

the presence of amylose is again indicated.

Subfractionation of the thymol-amylopectin was also visualised as being a possible means of positive identification of amylose as the contaminant. Table 6. 7 shows the properties of the seven subfractions obtained. The  $\beta$ -amylolysis limits are of the same order of magnitude as those found for the amylopectins and no trend is obvious. No blue staining fraction was isolated. Due to the relative insolubility of the fractions precipitated by alcohol, losses of polysaccharide on filtration were high. (See Experimental). Only fraction 6 was obtained in substantial yield, most of the fractions being obtained in yields of about 10 mg. Therefore, since the original 250 mg. of thymol amylopectin used contained only ca 16 mg. of amylose, this subfraction was probably lost on filtration.

Despite the lack of success in isolating amylose, the foregoing experimental results point to the presence of the linear starch component and the iodine-binding capacity of the thymol-amylopectin may justifiably be attributed to contaminating amylose. Another possibility which merits consideration, in view of the initial precipitation of the polysaccharide by thymol, is that the thymol-amylopectin possesses some chains which are much longer than the average. However, this postulate is not compatible with the heterogeneity of the thymol-amylopectin as evidenced by the results from ultracentrifugation.

The properties of the thymol-amylopectin are not consistent with its being a plant glycogen. It has also /

TABLE 6. 7.

Subfractionation of Thymol-Amylopectin.

Fraction	1	2	3	4	5	6	7
Yield (mg)	10	8	14	15	11	105	7
$\beta$ -limit %	54	51	52	51	54	53	56
$\lambda$ max. ( $m\mu$ )	550-560	550-560	550-560	550-560	550-560	550-560	550-560

\* Sample from tuber size 4 - 5 cm.

\* Percentage conversion into maltose on treatment with  $\beta$ -amylase.

also been suggested, (Banks and Greenwood, 1959) that this polysaccharide might be degraded amylopectin, but it is not readily explicable why the fractionation procedure degrades part of the amylopectin while not degrading the much less stable amylose component.

The thymol-amylopectin may also be regarded as undispersed granular material. In the present work, the cooled starch dispersion containing thymol was filtered through glass wool, with a view to removing residual granular particles, and it may be that the lower yields of thymol-amylopectin point to the success of the filtration being only partial. Thus the thymol-amylopectin could be undispersed granular material, though this is not thought to be likely.

It is suggested that the thymol-amylopectin is merely a subfraction of the total amylopectin present in the granule, contaminated by amylose which - because it is not precipitated by n-butanol - is most probably small in size. This conclusion prompts the question of why the thymol-amylopectin is precipitated by thymol; Banks and Greenwood (1959), suggested that the mechanism was one of coprecipitation, and, indeed, it is not unreasonable to suggest that the formation of helical chains by the amylose-thymol complex, in the presence of a preponderance of amylopectin, might be accompanied by a limited degree of molecular entanglement with the amylopectin.

SECTION 6. II.

A Study of the Properties of Starches from  
Granules of Different Sizes.



(a) INTRODUCTION.

The results from the previous section have shown that both the properties of the granule and the fine-structure of the starch components are dependent on the size of the granule. This statement holds for starches extracted from potato tubers of different sizes, where the number-average granular diameter increases as the tuber size increases. Ideally, however, the granules of different sizes should originate from the same sample of starch. Similar information could then be obtained without the uncertainties introduced by using tuber size as an initial criterion of maturity. Such fractions would also possess the additional advantage of having much narrower distributions of granule sizes.

Higginbotham and Richardson (1940), separated a sample of potato starch, by elutriation in water, into fractions of uniform granule size ranging from  $6\mu$  to  $29\mu$ . On the one hand, the phosphorus contents of the granules were found to increase with decreasing radius; on the other hand, the reducing powers, specific viscosities and solubilities of the fractions in phosphate buffer solution were almost identical, while the differences in swelling capacity in the same buffer were small. It was concluded that the average chain-lengths were the same and "that the granular structure factor was common to all granules."

(b) EXPERIMENTAL.

A sample of starch, (30g; sample No.6) was chosen from the centre of the range used for growth studies in the preceding section, and separated into five fractions using the method of Decker and Hoeller (1962), in which the granules were allowed to sediment through distilled water for varying lengths of time. Using 400 ml. 'tall' beakers and a maximum sedimentation height of 10 cm., the sedimentation times found most convenient were 4, 8, 12, 16 and 20 minutes. The process was started by stirring the 'total' starch in distilled water in beaker No. 1, and allowing the suspension to sediment for 4 minutes, after which the un-sedimented portion was poured into beaker No. 2. In subsequent operations the second beaker was allotted a sedimentation time of 8 minutes and at the same time as starch was sedimented in beaker No. 2 for 8 minutes, the sediment in beaker No. 1 was restirred and again allowed to sediment for 4 minutes. In general, after sedimentation in beaker  $n$  for time  $t_n$ , the supernatants were poured into beaker  $n + 1$  and sedimented for a time  $t_{n+1}$ , where  $t_{n+1} > t_n$ . Having gone through this procedure till the fifth beaker was reached, the result, from microscopic observation, was a fairly narrow distribution of the small granules, but a much wider distribution of the larger granules. The sediments for times 4 and 8 minutes were, therefore, recombined and put through the whole procedure again, in a separate set of beakers. /

beakers. The fractions obtained were then combined with the corresponding fractions obtained earlier.

Since, at this stage, the granules in the beakers with the longer sedimentation times had undergone fewer sedimentations, the procedure was repeated, first omitting beaker No. 1 and then subsequent beakers, until all the samples had been subjected to an identical number of sedimentations.

#### Fractionation and Characterisation of the Granules.

Fractionation of the granules and characterisation of the granules and starch components, with respect to gelatinisation temperature, iodine affinity, number average granular diameter, limiting viscosity number,  $\beta$ -amylolysis limit,  $\beta + \alpha$  amylolysis limit and length of unit chain, was carried out as in the previous section.

#### (c) RESULTS AND DISCUSSION.

The properties of the sedimented granules are shown in Table 6. 8.

TABLE 6. 8  
Properties of the Sedimented Granules.

Sam- ple No.	Sedi- ment- ation Time (Mins)	Yield (Wt.%)	No. Av. Granule Diameter ( $\mu$ )	Gelatin- isation Tempera- ture ( $^{\circ}$ C)	Iodine Affinity	Amylose (%) <sup>/</sup>
Total Starch	-	-	28	63 - 64	3.35	18.2
1	4	28	37	62 - 63	3.8	19.5
2	8	38	28	63 - 64	3.5	18.0
3	12	21	16	64 - 65	3.25	16.7
4	16	6	10	65 - 66	3.1	16.0
5	20	4	7	67 - 68	2.8	14.4

<sup>/</sup> Calculated from iodine affinity.

The sedimentation process has yielded fractions, of narrow size /

size distribution, varying in number-average granule diameter from 7 to  $37\mu$ . Fig. 6. 7 shows the distribution of the fractions in comparison with the parent sample of starch (dotted line) and photomicrographs (magnification x 200) of some of the starches are shown in Fig. 6. 8.

The trends exhibited by the granular properties verify those found in the preceding section, namely, as granular size increases there is an increase in the ratio amylose:amylopectin and a corresponding reduction in the gelatinisation temperature of the granules. The import of these variations has already been discussed.

The characteristics of the starch components, shown in Table 6, 9, are clearly seen to be average values dependent on the distribution of granule sizes present in the starch sample.

TABLE 6. 9.

Properties of the Starch Components.

Sample	<u>Amyloses</u>			Iodine Affinity	% Purity	<u>Amylopectins.</u>		
	$[\eta]$	$\beta^{\#}(i)$	$\beta^{\#}+Z(ii)$			$\beta$ -limit <sup>*</sup> (i)	Chain Lgth.	Internal Chain Lgth.
Total Starch	345	87	101	0.06	99.7	53	23	8
1	400	85	98	0.07	99.6	52	21	8
5	310	91	101	0.04	99.8	59	25	8

\* Percentage conversion into maltose on treatment with (i)  $\beta$ -amylase (ii)  $\beta$ -amylase and Z-enzyme.  
 † Determined in M KOH at 25°C.

Thus the original value of 345 for the limiting viscosity number of the amylose is compounded from values ranging between 310 and 400. These figures do not represent the extreme /

FIG. 6.7.

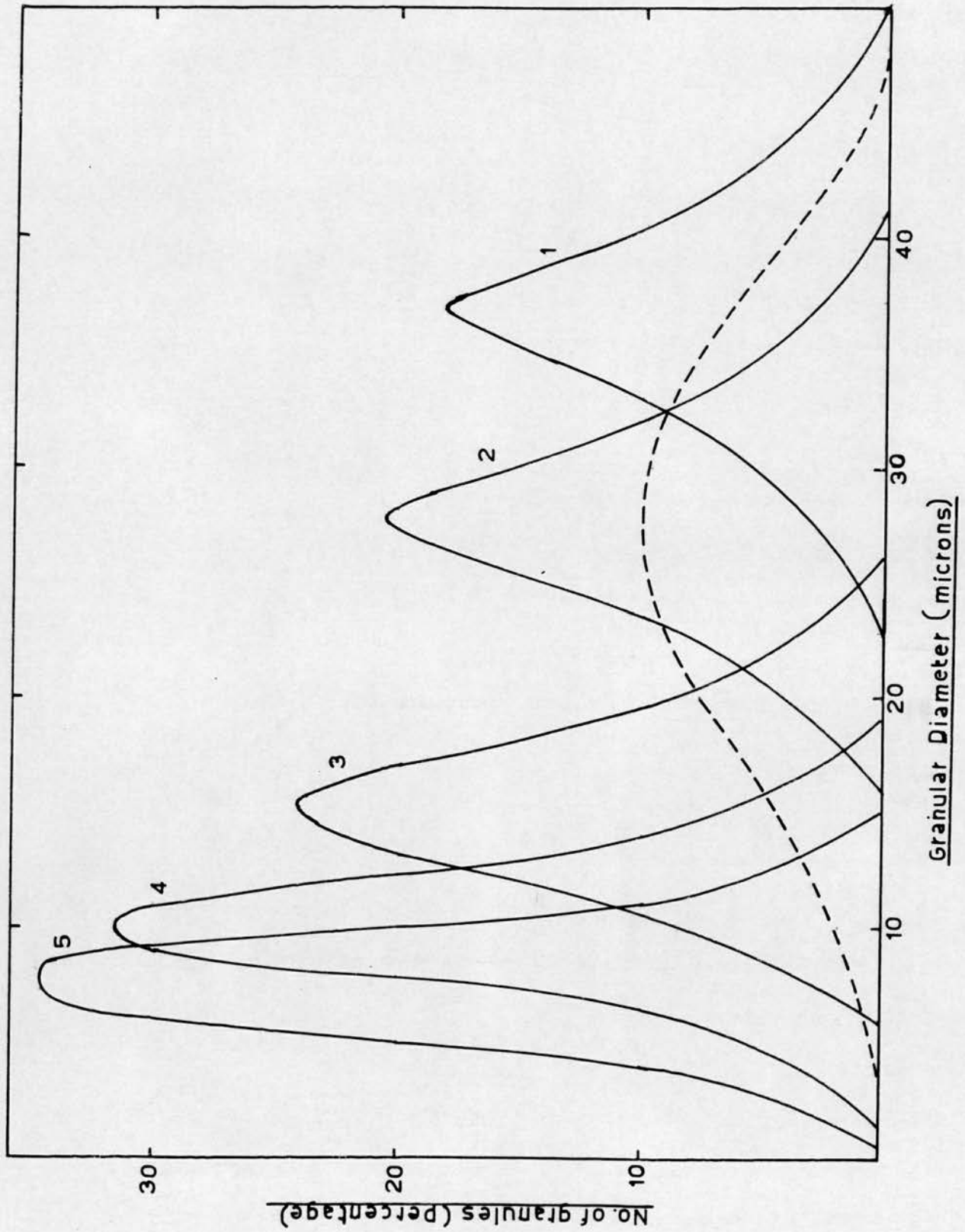
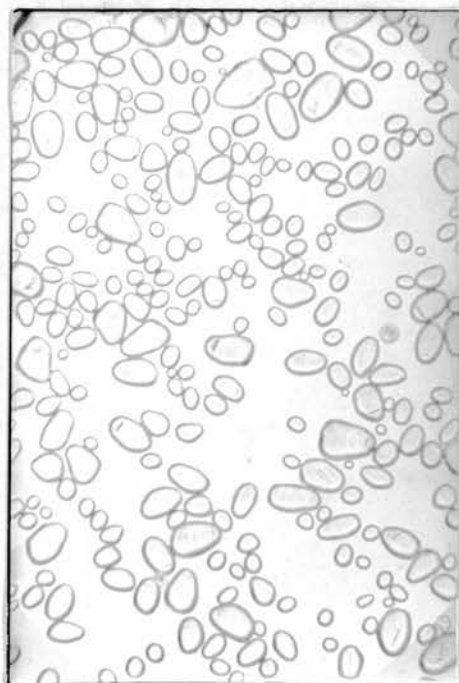




FIG. 6.8.

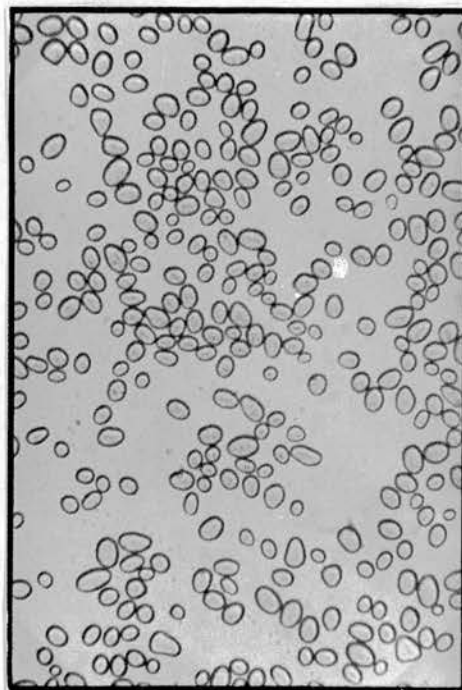
Total Starch



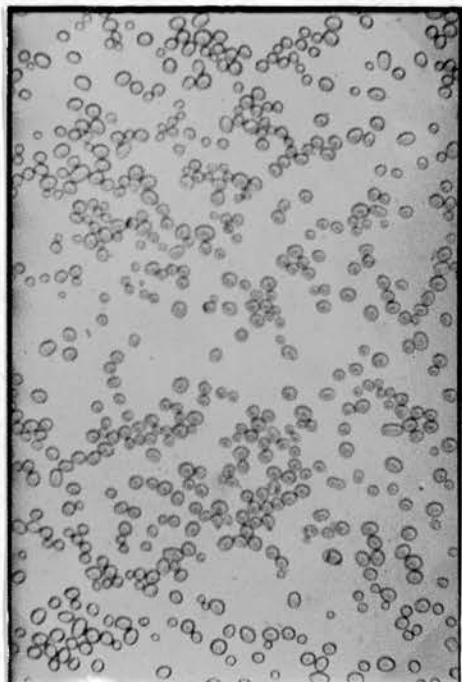
Fraction 1.



Fraction 3.



Fraction 5.



values of limiting viscosity number as granules both smaller and larger than the average values quoted for samples 1 and 5, were present in the total starch.

Similarly all the other characteristics of the components are seen to represent average values and thus it becomes necessary for the characteristics of the components to be accompanied by a value for the size of the granules from which the components have been obtained.

It may also be seen from Table 6. 9 that the fine-structure of the starch components varies with granule size, even when the granules have all been isolated from the same size of tuber. The  $\beta$ -amylolysis limit of the amylose component decreases with increasing granule size, while the  $\beta$ -amylolysis limit and the chain-length of the amylopectin decrease with increasing granule size. The trends in fine-structure thus verify the findings of the previous section.

### GENERAL DISCUSSION.

The major prerequisite of any theory for starch biosynthesis is that it should be able to explain how, (i) two widely differing polysaccharides can be synthesised in the same environment, (ii) how amylose and amylopectin can be deposited and arranged in the granule.

Considering current theories in the light of the foregoing results, relatively few precise distinctions can be made. The concurrent biosynthesis of amylose and amylopectin must necessarily presuppose that some form of barrier, or degree of separation, exists between the enzyme systems responsible; in particular, the access of the branching enzyme to the linear amylose molecules must be restricted.

Whelan (1958) postulated the existence of a semipermeable membrane separating the branching enzyme from the growing amylose molecule. However, recent electron microscopic studies (Badenhuizen, 1964) indicate that, in fact, no such barrier exists in the amyloplasts which are the centre of starch biosynthesis. In addition, such a mechanical barrier would entirely exclude the possibility of branching, however limited, in amylose. The growth-study results show that there is present in amylose a barrier to  $\beta$ -amylolysis, the amount of which increases with increase in granule size. Although the precise nature of this barrier has not been determined, the possibility of branching merits serious consideration and such a possibility must, therefore, also be considered as an operative factor in biosynthesis. The net result of the system proposed by Whelan /

Whelan is the incorporation of three glucose units into amylopectin and one glucose unit into amylose. There is consequently little scope for variation in the ratio of amylose to amylopectin in the granule and there would appear to be no reason why, in a system of this type, the ratio should gradually change as the granule grows. By Whelan's theory, the amylose and amylopectin are considered to migrate from the centre of biosynthesis; the major objection to this postulate is that the synthesising enzymes can equally well migrate. In this case, branching might be expected to occur in the amylose, although it is questionable whether the components in the granular state would act as primers for further synthesis, at least by the enzyme systems postulated by Whelan. On the other hand, it is difficult to visualise an increase in the degree of branching of the amylopectin component with growth, unless the branching enzyme is associated with the deposited amylopectin and capable of further synthesising activity.

Considering Erlander's theory (1958), whereby enzymic debranching of a glycogen-type molecule is postulated, the process of debranching would be expected to lead to an increase in the length of the amylopectin chains. It is therefore difficult to visualise how a debranching enzyme could act on a glycogen molecule to result in a structure which is, as has been observed, becoming increasingly branched. However, the major objection to this theory is that no debranching enzyme of the type postulated has yet been discovered /

discovered. In addition, it might reasonably be expected that if glycogen were an intermediate in starch synthesis its widespread occurrence in plants would have been well established; on the contrary, glycogen has been identified in only one plant source - Zea mays. In the light of present knowledge, it does not seem probable that starch biosynthesis involves a glycogen-type intermediate.

The recent researches of Leloir and coworkers on the sugar nucleotides has led to a resurgence of interest in the general field of starch biosynthesis. The discovery of starch synthetase, or perhaps more correctly amylose synthetase, has provided new information indicating that amylose and amylopectin may be, indeed, synthesised by separate enzyme systems. This postulate has resulted from the observation of Leloir and coworkers that a starch synthetase preparation acted on a uridine diphosphate glucose (U.D.P.G.) substrate to give preferential incorporation of glucose into the amylose component of the granule. Amylopectin is still generally regarded as being synthesised by phosphorylase and a branching enzyme.

A dual pathway system of the above kind readily explains some of the results observed in studying the growth of the potato granule. For instance, the virtual absence of phosphorus in amylose and its presence in amylopectin can be explained; in addition, the extreme divergence in the nature of these two polysaccharides can be more easily understood on the basis of different metabolic pathways. /



pathways. Since the granule itself can act as a precursor for further synthesis - however limited this may at present be considered - it is possible to visualise a lengthening of the amylose molecule by this type of mechanism. As the amylose synthetase is indubitably attached to, or perhaps even embedded in, the granular structure (Whelan, 1963), this postulate would involve the diffusion of the sugar nucleotide into the granule. Larger granules would present a greater surface area for such diffusion and so this factor could perhaps contribute to the observed increase in the ratio of amylose: amylopectin with growth of the granule. However, while such a mechanism is certainly operative, factors such as precursor availability, affecting the various enzymic equilibria, are much more likely to be responsible for the variation in the relative amounts of amylose and amylopectin.

Despite the advantages of the above theory, it is nevertheless still necessary to suppose that the branching enzyme is prevented from acting on amylose. Whelan (1963) suggested that the barrier may even be amylopectin. It was postulated that the enzyme synthesising amylose became surrounded by amylopectin preventing access to the branching enzyme but not to the sugar nucleotide substrate. Amylose synthesis was therefore thought to take place within a core of amylopectin. Badenhuizen (1964) on the other hand has suggested that the formation of a complex between the amylose and the amylose synthetase may, effectively, act as a barrier to the action of  $\alpha$ -enzyme.

The /

The mechanism of starch deposition is thought (Badenhuizen, 1963) to be one of crystallisation of the granular material from a coacervate. Growth takes place largely by apposition but intussusception can also occur. This latter type of mechanism is necessary to account for the incorporation of glucose by the starch synthetase enzyme mentioned above.

Although the question of which pathway of starch synthesis - phosphorylase or synthetase or both - operates in vivo, cannot be settled by the observations on granular growth, these studies have provided a set of data which must, along with many other factors, be comprehensively explained before any theory for starch biosynthesis can be regarded as being correct. The more recent researches concerning starch synthetase are more promising, both from a theoretical and factual viewpoint. On the other hand, these developments emphasise the need for much further research before the extremely complex problem of starch biosynthesis can be solved.

SECTION 7.

THE EFFECT OF HIGH - ENERGY ELECTRONS ON  
POTATO STARCH AND ITS COMPONENTS.

(a) Introduction.

The general field of radiation chemistry of the carbohydrates has been covered in a review by Phillips (1961). However, irradiation of polysaccharides and, in particular, of starch was given only limited attention.

Irradiation phenomena may result from the use of either ultra-violet radiation, X-rays or gamma radiation. Absorption of ultra-violet radiation depends, in general, on the molecular structure and only indirectly on atomic composition. The energy of X- and gamma-rays is almost entirely absorbed by ejecting electrons from the atoms through which they pass and this process is entirely independent of the manner in which these atoms are combined into molecules. X-rays are thus not very selective in action. Essentially, there is no difference between the effects produced by fast electrons and by X-rays, except that the range of the former is small, while the latter can penetrate much further and release electrons in the interior of the irradiated sample. In general, the single atom initially affected by the radiation makes a negligible contribution to the total chemical change, which arises mainly from the ionisation and excitation processes initiated by the secondary, high-speed electrons.

Irradiation of starch has been achieved by use of ultra-violet radiation (Samec, Kosovinc and Reuschl, 1958), gamma-rays (Oreshko et al, 1960; Oreshko and Korotchenko, 1958), X-rays (Ehrenberg et al, 1957),  $\beta$  -rays (Radley, 1960; Samec, 1961) and ultrasonic radiation (Samec et al, 1958).

The /

The predominant feature emerging from studies on the irradiation of starch is that degradation occurs. This is generally inferred from the decrease in viscosity of solutions of the polysaccharide (Takaoka et al, 1960; Mishina and Nikuni, 1959; Khenokh, 1950) and from the formation of reducing substances (Khenokh, 1947; Kersetz et al, 1959). At the same time, small fission products may be formed, e.g. dextrans, glucose, maltose, maltotriose and sugar acids, (Mishina and Nikuni, 1959; Khenokh, 1947). Irradiated starch solutions have also been found to develop an absorption maximum at ca 265 m ; this has been attributed to the formation of dihydroxy acetone, (Oreshko and Korotchenko, 1960; Khenokh, 1955; Khenokh, 1957). In addition the formation of volatile gaseous products such as hydrogen, carbon monoxide, carbon dioxide and methane has been reported, (Oreshko et al, 1962; Mishina and Nikuni, 1959; Dilli and Garnett, 1963).

Similar degradative phenomena occur when the starch components are irradiated in either the solid state, (Doguchi et al, 1961; Dilli and Garnett, 1963) or in solution (Whelan and Peat, 1950; Bourne, Stacey and Vaughan, 1956).

Water exerts a protective influence in the radiative degradation of starch. It has been suggested (Oreshko, 1960) that this protective action may be associated with the formation of hydrogen bonds between the functional groups of a glucose ring and the water molecules. Ehrenberg et al (1957) supposed that "water exerts its maximum protection at high water-contents (ca 20%) by eliminating the chemical rearrangements following excitation /



excitation, whereas the chemical consequences of ionisation are not affected to the same degree."

Bourne et al (1956), from results on the irradiation of amylose solutions, indicated that oxygen inhibits the degradation. Production of acid was found to be a function of the time of exposure to the radiation rather than of the extent of degradation of the amylose. Thus at least two independent reactions, one causing degradation and the other leading to the production of acid, were proceeding simultaneously.

Despite the amount of research done, in the past two decades, on the irradiation of starch, very few workers have fractionated the irradiated granules. Where fractionation has been carried out (Samec, 1960) it has been achieved by electrophoresis. This method, however, gives a slow, incomplete fractionation and is accompanied by considerable retrogradation. In the following work, therefore, the irradiated starches have been fractionated by the more conventional method of selective precipitation of the amylose component from a dispersion.

(b) Experimental.

Materials.

The irradiated samples of commercial potato starch were obtained by courtesy of Mr. J.A. Radley, of the J.A. Radley Research Institute, Berkshire, England. Irradiation was carried out using a 2-me V. Van der Graaf generator; doses ranging from  $5 \times 10^4$  to  $10 \times 10^6$  rads. were administered as follows:- Flat packets of starch were passed under a  $\frac{1}{3}$  inch wide strip of electrons at 20 inches per minute, so that the dosage rate worked out at  $1.8 \times 10^9$  rads./hr. This was carried out in an atmosphere of nitrogen. Irradiation was almost entirely by electrons, but inevitably, there was a contribution ( $10^{-4}$  -  $10^{-5}$  of total dose) from X-rays, due to scattering from the steel conveyor. The temperature did not rise appreciably during irradiation.

Characterisation and Fractionation of the granules.

The acidity of the samples was determined by potentiometric titration of a suspension of starch (lg. in 10 ml. of water), in a nitrogen atmosphere, with 0.01 M sodium hydroxide. The gelatinisation temperatures and iodine affinities of the granules were determined as detailed in Section 2.

The method of fractionation differed slightly from the more usual procedure (see Section 3); in view of the acidity of the samples, they were dispersed in boiling water containing 4 ml. of phosphate buffer per litre (pH 6.4; 20% phosphate i.e. 16.4% with respect to anhydrous potassium dihydrogen phosphate and 3.6% with respect to anhydrous dipotassium hydrogen phosphate; Radley, 1953). The amylose was first precipitated using thymol and /

and then recrystallised with n-butanol. The supernatant liquor containing the amylopectin was extracted with ether, concentrated on a rotary-evaporator (temperature  $< 35^{\circ}\text{C}$ ) and the resultant solution dialysed against distilled water, prior to the recovery of the amylopectin by freeze drying.

#### Characterisation of the Components.

The extents of conversion of the amylose samples into maltose were measured for (i) the action of pure  $\beta$ -amylase and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme. Limiting viscosity numbers in M potassium hydroxide were determined at  $25^{\circ}\text{C}$ .

The iodine affinity of the amylopectins, the extent of conversion into maltose under the action of pure  $\beta$ -amylase and the average length of unit chain were estimated as detailed in Section 2.

Sedimentation coefficients were measured in aqueous solution using a Spinco, Model E, ultracentrifuge. These measurements were kindly carried out by Dr. C.T. Greenwood.

The optical density of solutions of the amylopectins (5 mg./100 ml.) stained with iodine (0.002% iodine in 0.02% potassium iodide) was measured in 1 cm. cells in a Unicam spectrophotometer, Model SP 600.

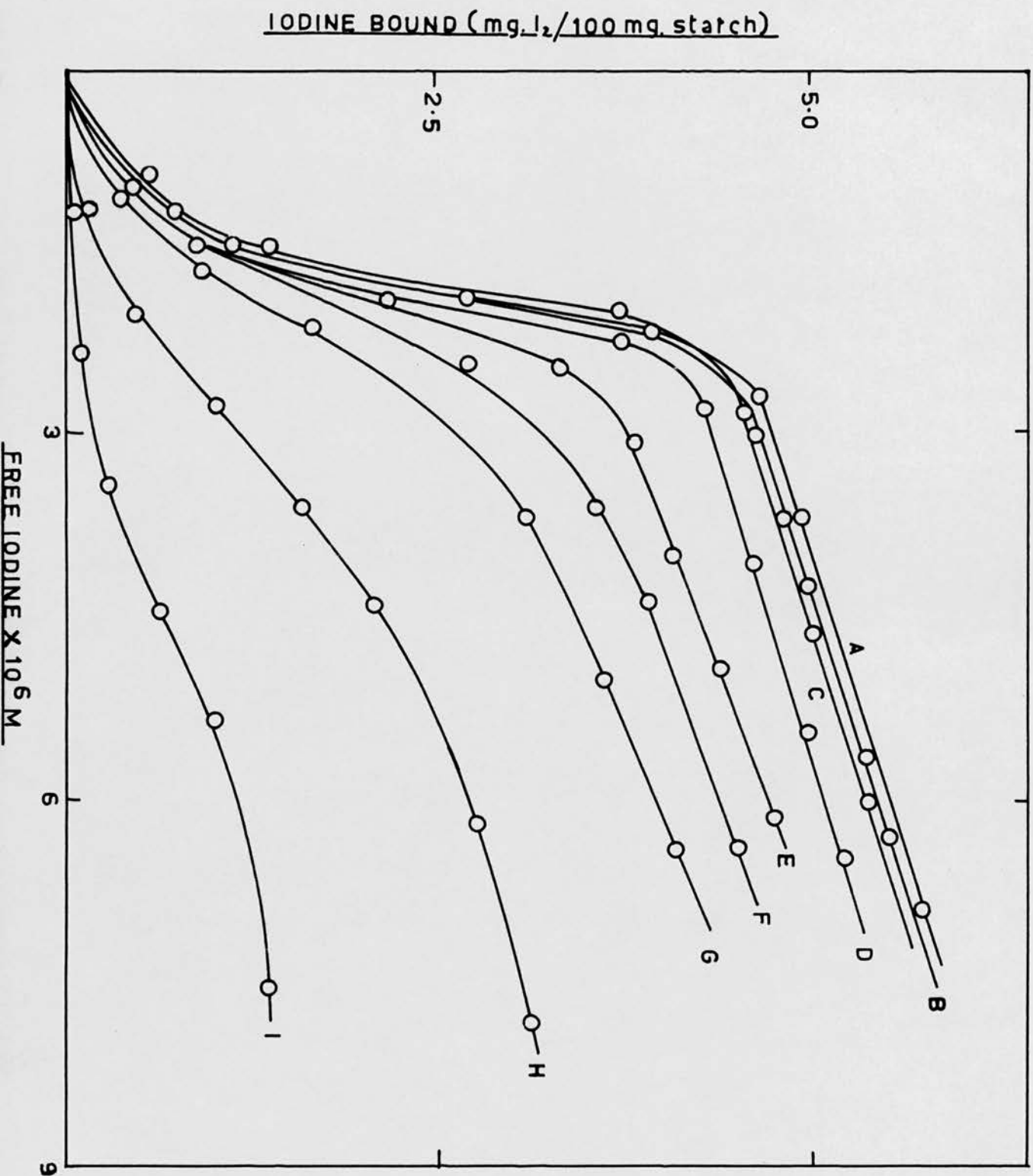


FIG. 7.1.

(c) Results and Discussion.

Properties of the Granular Starches.

The acidity of the starches, shown in Table 7. 1, was appreciably less than that found by Radley (1960), for samples which had received higher doses of high-energy electrons. For doses larger than  $10^6$  rads., the starches were a creamy-yellow colour; this colour deepened with increasing radiation dose. There was no obvious damage to the granular structure and microscopic observation also showed that all the starches exhibited birefringent properties. Furthermore, the behaviour on gelatinisation and the gelatinisation temperatures were not appreciably altered except for samples G and I. However, gelatinisation is not a sensitive measure of granular modification, for quite profound differences were apparent when the iodine-binding power of the samples was measured. The results of these experiments are shown in Fig. 7. 1. It can be seen that as the radiation dose increases, there is a lowering in the iodine-binding power of the starch. The changes induced in samples B - D appear to be identical to those reported by Cowie and Greenwood (1957) for the acid degradation of potato starch granules. For samples E - I, the modification of the starch is so profound that the usual sigmoidal adsorption isotherm is not found and, moreover, the concentration of free iodine necessary to saturate the samples increases. This indicates a decrease in the length of the linear material present. The usual procedure of extrapolating the linear portion of the adsorption curve to zero free iodine /



TABLE 7.1

Properties of Irradiated Granular Starches.

Sample	Irradiation dose (rads. $\times 10^{-6}$ )	Acidity (equivs./g. $\times 10^6$ )	Gelatinisation temperature ( $^{\circ}$ C)	Iodine affinity <sup>1)</sup>	"Amylose" (%) <sup>2)</sup>
A	0	4.4	68-69	4.5	23
B	0.05	6.0	-	4.4	22.5
C	0.1	7.4	67-69	4.3	22
D	0.2	8.5	-	4.1	21
E	0.5	10.3	67-69	3.6	18.5
F	1	12.7	-	3.4	17.5
G	2	17.9	64-65	3.1	16
H	5	29.0	-	2.4	12
I	10	40.0	60-61	1.1	6

1) Expressed as mg. of  $I_2$  bound per 100 mg. of starch. Derived from Fig. 7.1 as described in Text.

2) Apparent amylose-content. Calculated from iodine affinity.

iodine concentration, in order to obtain the iodine affinity, is not applicable in these cases. An approximate value for the iodine affinity was obtained, therefore, from the point of intersection of the rising binding portion of the curves and the linear part. The resultant values are shown in Table 7. 1, although those for samples H and I are approximate. Apparent values for the amylose contents shown in the last column of Table 7. 1 were arbitrarily calculated from  $\% \text{ Amylose} = \frac{\text{iodine affinity}}{19.5} \times 100$ .

The shape of the iodine-binding curves for samples E - I indicates a loss of iodine-binding material and suggests that the amylose component of these starches was extensively degraded.

Through the courtesy of Mr. D.J. Bryce, the irradiated starches were pyrolysed in vacuo at 300°C and the decomposition products examined by gas-chromatography (Bryce and Greenwood, 1963). The chromatograms obtained were found to be identical to those for non-irradiated starches and so the presence of any small-molecular-weight fission products did not appreciably alter the pyrolysis "cracking-pattern" of the polymer.

#### Properties of the Components.

The properties of material, which was precipitated with conventional precipitating agents for amylose, are shown in Table 7. 2. The enzymic experiments show that only samples B - D, of the irradiated starches, contained amylose in the sense that they were incompletely degraded by pure  $\beta$ -amylase, while /

TABLE 7. 2.

Properties of Amylose Components from Starches.

Sample <sup>1)</sup>	$\beta$ -Limit (i). <sup>2)</sup>	$\beta$ -Limit (ii) <sup>2)</sup>	$[\eta]$	$\overline{D.P.}$ <sup>3)</sup>
A	83	100	230	1700
B	86	102	220	1650
C	88	99	150	1100
D	88	98	110	800
E	87	92	95	700
F	86	91	80	600
G	84	89	50	350
H	83	86	40	300
I	83	87	35	250

1) As in Table 7. 1.

2) Conversion into maltose under the action of (i) pure  $\beta$  -amylase, and (ii)  $\beta$  -amylase and Z-enzyme

3) Number-average degree of polymerisation; calculated from  $\overline{D.P.} = 7.4 \times [\eta]$ .

while being completely degraded by a mixture of  $\beta$ -amylase and Z-enzyme. The radiation did, in fact, introduce modifications into the  $\alpha$ -1:4 glucan structure, since the extent of degradation by  $\beta$ -amylase was reduced. For samples E - I, although the  $\beta$ -amylolysis limits were altered, some other type of structural modification had occurred, as the polysaccharide was incompletely degraded by the mixture of  $\beta$ -amylase and Z-enzyme. It is not thought likely that this could be due to contaminating, degraded amylopectin.

The decrease in value of the limiting viscosity number for the amylose samples, as shown in Table 7.2, indicates that extensive depolymerisation was occurring, with increasing dose of irradiation. The extent of this is illustrated in Fig. 7. 2 (Curve 1). Approximate values of the degree of polymerisation of the samples are given in the last column of Table 7. 2.

In the case of the amylopectin samples, the method of isolation would necessitate that they would contain any soluble, degraded, amylose-type material. However, there is no evidence for this. The iodine-binding power of the amylopectins from irradiated samples was less, in fact, than that for the control sample A, as shown by the typical results in Fig. 7. 3. This is in contrast to the behaviour of amylopectins from acid-degraded starch (Cowie and Greenwood, 1957). Again, the optical density of iodine-complexes showed a maximum absorption at about  $550 \text{ m}\mu$ , with no evidence of a peak at higher wavelengths corresponding to linear material. Typical results are shown in Table 7. 3.

Profound depolymerisation and structural changes were apparent /

FIG. 7.2.

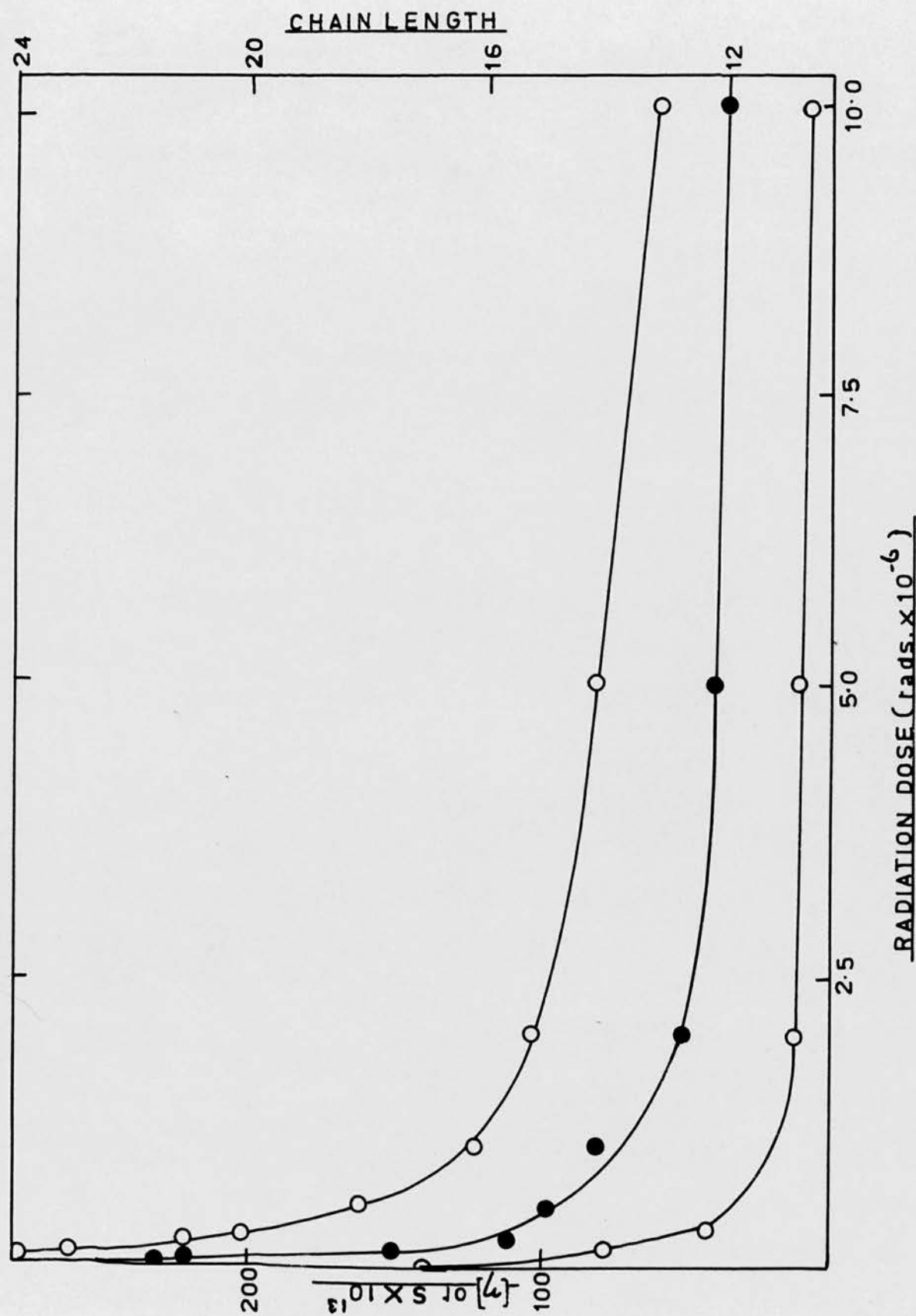




FIG. 7.3.

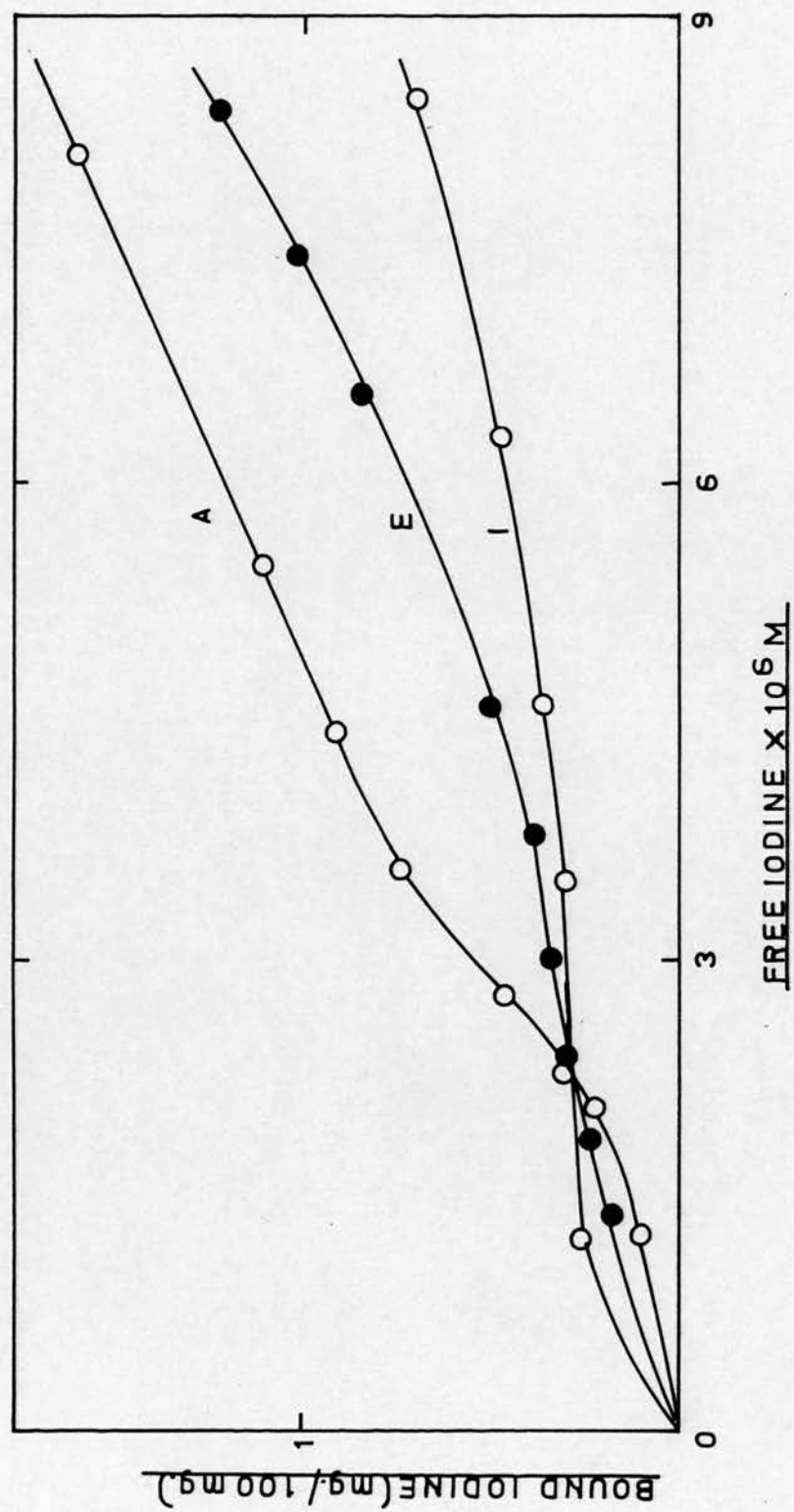


TABLE 7. 3.

Optical Density<sup>1)</sup> of Iodine-complex of Some Amylopectins.

Sample	Wavelength (m $\mu$ )											
	460	480	500	520	540	560	580	600	620	640	660	680
A	0.217	0.248	0.268	0.304	0.332	0.333	0.316	0.287	0.255	0.235	0.193	0.164
E	0.191	0.219	0.251	0.288	0.314	0.319	0.297	0.265	0.235	0.200	0.184	0.150
I	0.252	0.280	0.319	0.356	0.376	0.370	0.334	0.293	0.255	0.215	0.180	0.130

1) For conditions, see Text.

apparent in the amylopectin components. The samples were very readily soluble in cold water, indicating a reduction in molecular size. This was confirmed by ultracentrifuge measurements, when the sedimentation coefficient was found to decrease rapidly with irradiation dose, as shown in Table 7. 4 and also in Fig. 7. 2 (Curve 2). Simultaneously, as well as depolymerisation of the main chain, breaks must also have occurred in the branches, as indicated by changes in the average length of unit-chain (Table 7. 4). The extent of this, in relation to the radiation dose, is shown in Fig. 7. 2 (Curve 3).

-amylolysis limits decreased with increase in radiation dose, indicating that the irradiated polymer was becoming increasingly less amenable to enzymic degradation. This also indicates the formation of structural modifications. In general, it is concluded that the action of relatively small doses of high-energy electrons results in profound degradation and structural modification of the starch components. However, much larger doses are required before gross modification of granule structure is evident.

TABLE 7. 4.

Properties of Amylopectin Components from Starches.

Sample <sup>1)</sup>	$\beta$ -Limit <sup>2)</sup>	Av. chain-length.	Length of internal-chain <sup>3)</sup>	Sedimentation Coefficient x 10 <sup>13</sup>
A	58	24	8	140
B	57	23	7	120
C	55	23	7	105
D	54	21	7	72
E	51	18	6	42
F	49	16	6	20
G	19	15	5	12
H	48	14	5	10
I	46	13	5	8

1) As in Table 7. 1.

2) Conversion into maltose under the action of pure  $\alpha$ -amylase.

3) Calculated from  $\left\{ \text{chain-length} - \left[ \frac{(\text{chain-length} \times \beta\text{-limit})}{+ 2.5} \right] \right\}$

### SUMMARY.

After a brief survey of current concepts in the chemistry of starch and its components, the physical and enzymic techniques used in this work have been discussed in detail.

Potato starch has been fractionated by a chloral hydrate method; the products obtained were comparable to those obtained by more conventional methods of fractionation. The efficiency of polar complexing agents for separating amylose has been investigated; n-butanol and n-amyl alcohol have been found to be preferable. Hydrophobic complexing agents have also been used to fractionate starch. Pure amylopectin fractions were readily obtained and amyloses of a high degree of purity were obtained on recrystallisation.

The effect of a commercial extraction procedure on the fine structure of maize starch has been investigated. Limited degradation of the amylose component was found to occur. The fine structure of the amylopectin from high amylose-content maize starch has been examined in detail; the apparent long chain-length of amylo-maize amylopectin has been shown to be due to contamination by short-chain amylose.

An investigation of the starch from the fruit of the potato showed that the unusually prominent granular markings were not coincident with gross changes in the granular structure.

A study of the properties of starches from the growing potato tuber showed that the gross properties both of starch and the components are a function of granule size. Profound changes in the /



the fine-structure of the components also take place. The amylose component was shown, by viscosity and sedimentation measurements, to increase in molecular size; the  $\beta$ -amylolysis limit, however, decreased with increase in granule size. The molecular weight of the amylopectin also increased and measurements of chain-lengths and  $\beta$ -amylolysis limits indicated that the structure was becoming increasingly branched. Current theories of the biosynthesis of starch have been reviewed and discussed in terms of the results obtained.

High-energy electrons, administered in various doses have been shown to degrade potato starch and modify the structure of the components.

B I B L I O G R A P H Y.

- ALEXANDER, B.H., R.J. : J. Amer. Soc., 73, 4658.  
DIMLER and  
C.L.MEHLTRETTER (1951).
- ANDERSON, D.M.W., and  
C.T.GREENWOOD (1955) : J. Chem. Soc., 3016.
- ANDERSON, R.A.,  
C.VOJNOVICH and  
G. SOEDOMO (1963) : Stärke, 15, 355.
- BADENHUIZEN, N.P.(1955): Protoplasma, 45, 315.
- BADENHUIZEN, N.P.(1959): Protoplasmatologia, Band II, Cytoplasma, 1.
- BADENHUIZEN, N.P.(1961): Recent advances in Botany, Section 11, 1258.
- BADENHUIZEN, N.P.(1963): Nature, 197, 464.
- BADENHUIZEN, N.P.(1964): Personal communication.
- BADENHUIZEN, N.P. and  
R.W.DUTTON (1956): Protoplasma, 47, 156.
- BANKS, W. and  
C.T.GREENWOOD (1959a): Biochem. J., 73, 237.
- BANKS, W. and  
C.T.GREENWOOD (1959b): J. Chem. Soc., 3436.
- BANKS, W. and  
C.T.GREENWOOD (1963) : Adv. Carbohydrate Chem., 18, 357.
- BANKS, W., C.T.GREENWOOD  
and J.THOMSON (1959) : Chem. and Ind., 928.
- BANKS, W., C.T.GREENWOOD  
and I.G.JONES (1960) : J. Chem. Soc., 150.
- BARTLETT, G.R. (1959) : J. Biol. Chem., 1, 466.
- BATES, F.L., D.FRENCH  
and R.E.RUNDLE (1943): J. Amer. Chem. Soc., 65, 142.
- BEAR, R.S., (1944) : J. Amer. Chem. Soc., 66, 2122.
- BERSOHN, R. and  
I.ISENBERG (1961) : J. Chem. Phys., 35, 1640.
- BICE, C.W., M.M.MACMASTERS  
and G.E.HILBERT(1945): Cereal Chem., 22, 463.
- BOBBITT, J.M. (1956) : Adv. Carbohydrate Chem., 11, 1.

- BOURNE, E.J., G.H. : J. Chem. Soc., 1687.  
DONNISON, N.HAWORTH and  
S.PEAT (1948).
- BOURNE, E.J., M.STACEY  
and G. VAUGHAN (1956) : Chem. and Ind., 573.
- BROWN, F., T.G.HALSALL,  
E.L.HIRST and  
J.K.N.JONES (1948) : J. Chem. Soc., 27.
- BRYCE, D.J. and  
C.T. GREENWOOD (1963) : Stärke, 15, 166, 285.
- BUTTROSE, M.S. (1962) : J. Cell Biology, 14, 159.
- BUTTROSE, M.S. (1963) : Stärke, 15, 85.
- CHEN JR., P.S.,  
T.Y.TORIBARA and  
H.WARNER (1956) : Analyt. Chem., 28, 1756.
- CINCOTTA, J.J. (1960) : J. Agric. Food Chem., 8, 145.
- CLARK, C.F. (1921) : U.S. Dept. of Agric. Bull, 958. via  
Hector, J.M. "Introduction to the Botany  
of Field Crops." Vol. II, Non Cereals.  
Central News Agency, Johannesburg.
- COWIE, J.M.G. and  
C.T. GREENWOOD (1957a) : J. Chem. Soc., 2862.
- COWIE, J.M.G. and  
C.T. GREENWOOD, (1957b) : J. Chem. Soc., 2658.
- COWIE, J.M.G. and  
C.T. GREENWOOD (1957c) : J. Chem. Soc., 4640.
- DAVIS, W.E. and  
J.H. ELLIOT (1949) : J. Colloid Sci., 4, 313.
- DEATHERAGE, W.L.,  
M.M.MACMASTERS and  
C.E. RIST (1955) : Trans. Am. Assoc. Cereal Chemists, 13, 31.
- DECKER, P. and  
H.HOELLER (1962) : J. Chromatog., 7, 392.
- DE FEKETE, M.A.R.,  
L.F.LELOIR and  
C.E. CARDINI (1960) : Nature, 187, 918.
- DILLI, S. and  
J.L.GARNETT (1963) : Chem. and Ind., 409.

- DOGUCHI, M., Y.YOKOYAMA : Kogyo Kagaku Zasshi, 64, 2001. via Chem.  
and I.OKADA (1961) Abstr., 57, 7503b.
- DUBOIS, M., K.A.GILLIES,  
J.K.HAMILTON, P.A.REBERS  
and F.SMITH (1956) : Analyt. Chem., 28, 350.
- EHRENBERG, L., M.JAARMA  
and K.G.ZIMMER (1957) : Acta. Chem. Scand., 11, 950.
- ERLANDER, S.R. (1958) : Enzymologia, 19, 273.
- ERLANDER, S.R. (1960) : Cereal Chem., 37, 81.
- ERLANDER, S.R. and  
D.FRENCH (1958) : J. Amer. Chem. Soc., 80, 4413.
- FISKE, C.H. and  
Y. SUBBA ROW (1925) : J. Bio. Chem., 66, 375.
- FLORY, P.J. (1953) : "Principles of Polymer Chemistry," Cornell.
- FOGG, D.N. and  
N.T.WILKINSON (1958) : Analyst, 83, 406.
- FRENCH, D., A.O.PULLEY  
and W.J.WHELAN, (1963) : Stärke, 15, 349.
- FRENCH, D., and  
G.M. WILD (1953) : J. Amer. Chem. Soc., 75, 4490.
- FREY-WYSSLING, A. and  
M.S.BUTTROSE (1961) : Makromol, Chem., 44-46, 173.
- GIESEKING, G.A., H.J.  
SNIDER and C.A.GETZ  
(1935) : Ind. Eng. Chem., 7, 185.
- GILBERT, G.A. and  
J.V.R.MARRIOTT (1948) : Trans. Faraday Soc., 44, 84.
- GOODWIN, J.F., R.THIBERT,  
D.MACCANN and A.J.BOYLE  
(1958) : Analyt. Chem., 30, 1097.
- GREENWOOD, C.T. (1956) : Adv. Carbohydrate Chem., 11, 336.
- GREENWOOD, C.T. (1960) : Stärke, 12, 169.
- GREENWOOD, C.T. and  
J. THOMSON (1960) : Chem. and Ind., 1110.
- GREENWOOD, C.T. and  
J. THOMSON (1962a) : Biochem. J., 82, 156.

- GREENWOOD, C.T. and : J. Chem. Soc., 222.  
J. THOMSON (1962b)
- GRIFFITHS, W. (1892) : "The Principal Starches used as Food,"  
pages 59-60. Bailey and Son, Cirencester.
- HALSALL, T.G., E.L. HIRST,  
J.K.N. JONES and  
F.W. SANSOME (1948) : Biochem. J., 43, 70.
- HAMILTON, J.K. and  
F. SMITH (1956) : J. Amer. Chem. Soc., 78, 5910.
- HARRIS, G. and  
I.C. MACWILLIAM (1958) : Cereal Chem., 35, 82.
- HAWORTH, W.N., E.L. HIRST  
and F.A. ISHERWOOD  
(1937) : J. Chem. Soc., 577.
- HIGGINBOTHAM, R.S. (1949): Shirley Inst. Mem., 23, 171.
- HIGGINBOTHAM, R.S. and  
G.A. MORRISON (1948): Shirley Inst. Mem., 22, 141.
- HIGGINBOTHAM, R.S. and  
W.A. RICHARDSON (1940): Nature, 146, 234.
- HILBERT, G.E. and  
M.M. MACMASTERS (1946): J. Biol. Chem., 162, 229.
- HOBSON, P.N., W.J. WHELAN  
and S. PEAT (1950): J. Chem. Soc., 3566.
- HODGE, J.E., E.M. MONTGOMERY  
and G.E. HILBERT (1948): Cereal Chem., 25, 19.
- HOLLO, J., E. LASZLO, J. SZEJTLI  
and A. MANDI (1960): Stärke, 12, 351.
- HOLLO, J. and  
J. SZEJTLI (1960): Brauwissenschaft, 13, 380. via. Chem.  
Abstr. 55, 8301c.
- HOPKINS, R.H. and  
B. JELINEK (1948): Biochem. J., 43, 28.
- HUGGINS, M.L. (1942): J. Phys. Chem., 46, 151.
- I.U.P.A.C. (1952): J. Polymer Sci., 8, 257.
- JACKSON, E.L. and  
C.S. HUDSON (1936): J. Amer. Chem. Soc., 58, 378.



- JOHNSTON, J.P. and : Trans. Faraday Soc., 42, 789.  
A.G. OGSTON (1946)
- KERTESZ, Z.I., E.R.SCHULZ,  
G.FOX and M.GIBSON  
(1959) : Food Research, 24, 609.
- KHENOKH, M.A. (1947) : J. Gen. Chem. (U.S.S.R.), 17, 1024. via  
Chem. Abstr. 42, 3665 h.
- KHENOKH, M.A. (1950) : Zhur. Obshchei Khim., 20, 1560. via  
Chem. Abstr., 45, 963 d.
- KHENOKH, M.A. (1955) : Doklady Akad. Nauk, S.S.S.R., 104, 746. via  
Chem. Abstr. 50, 7603b.
- KHENOKH, M.A. (1957) : Trudy Pervogo Vsesoyuz. Soveshchaniya po  
Radiatsion. Khim., Akad. Nauk S.S.S.R.,  
182. via Chem. Abstr. 53, 12020a.
- KHENOKH, M.A., E.A.KUZICHEVA  
and V.F.EVDOKIMOV (1960): Doklady Akad. Nauk S.S.S.R., 135, 471. via  
Chem. Abstr., 55, 11316e.
- KIRKWOOD, J.G. and  
J. RISEMAN (1948): J. Chem. Phys., 16, 565.
- KJØLBERG, O. and  
D.J.MANNERS (1963): Biochem. J., 68, 258.
- LAMPITT, L.H., C.H.F.FULLER  
and L.COTON (1955): J. Sci. Food Agric., 6, 656.
- LANSKY, S., M.KOOI and  
T.J.SCHOCH (1949): J. Amer. Chem. Soc., 71, 4066.
- LARSON, B.L., K.A.GILLES  
and R.JENNESS (1953): Analyt. Chem., 25, 802.
- LEACH, H.W. and  
T.J.SCHOCH (1961): Cereal Chem., 38, 34.
- LELOIR, L.F. and  
C.E.CARDINI (1957): J. Amer. Chem. Soc., 79, 6340.
- LOWRY, O.H., N.R.ROBERTS,  
K.Y.LEINER, MEI-LING WU  
and A.L.FARR (1954): J. Biol. Chem., 207, 1.
- MacMASTERS, M.M., G.E.HILBERT,  
M.J.COX, J.W.ECK and  
C.W.BICE (1946): Quartermaster Corps Report, 17.

- MACWILLIAM, I.C., R.D. : J. Inst. Brewing, 62, 226.  
HALL and G.HARRIS(1956)
- MALAPRADE, L. (1928): Bull. Soc. Chim. France, 43, 683.
- MANNERS, D.J. and  
G.A.MERCER (1963): J. Chem. Soc., 4317.
- MATHESON, N.K., and  
J.M.WHEATLEY (1962): Austral. J. Biol. Sci., 15, 445.
- MATHESON, N.K., and  
J.M.WHEATLEY (1963): Austral. J. Biol. Sci., 16, 70.
- MAYWALD, E., R.CHRISTENSEN  
and T.J.SCHOCH (1955): Agric. and Food Chem. 3, 521.
- McGOURY, J. and H.MARK  
(1938): "Der Feste Korper," Liepzig, 103.
- MEYER, K.H. (1942): Adv. Colloid Sci., 1, 170.
- MEYER, K.H. and  
P.BERNFELD (1940): Helv. Chim. Acta, 23, 875.
- MEYER, K.H., P.BERNFELD,  
R.A.BIOSSONNAS,P.CURTLE  
and G.NOELTING (1949): J. Phys. Coll. Chem., 53, 319.
- MEYER, K.H., M.WERTHEIM and  
P.BERNFELD (1940): Helv. Chim. Acta, 23, 865.
- MISHINA, A. and Z.NIKUNI  
(1959): Nature, 184, 1867.
- MONTGOMERY, E.M. and  
F.R.SENTI (1958): J. Polymer Sci., 28, 1.
- MONTGOMERY, E.M., K.R.SEXSON  
and F.R.SENTI (1961): Stärke, 13, 215.
- MONTGOMERY, E.M., F.B.WEAKLEY  
and G.E.HILBERT (1949): J. Amer. Chem. Soc., 71, 1682.
- MUETGEERT, J. (1961): Adv. Carbohydrate Chem., 16, 299.
- MYRBÄCK, K. and L.G.SILLEN  
(1949): Acta Chem. Scand., 3, 190.
- NIELSEN, J.P. and P.C.GLEASON  
(1945): Analyt. Chem., 17, 131.
- NELSON, O.E. and H.W.RINES  
(1962): Biochem. Biophys. Res. Comm., 9, 297.

- O'BRIEN, J.A. (1951) : Agric. and Food Chem., 38, 684.
- ORESHKO, V.F. (1960) : Zhur. Fiz. Khim., 34, 2369.
- ORESHKO, V.F., L.E.CHERENKO  
and N.K.SHAKOVA (1960) : Doklady Akad. Nauk S.S.S.R., 134, 636.  
via Chem. Abstr. 55, 13976h.
- ORESHKO, V.F., L.F.GORIN  
and N.V.RUDENKO (1962) : Russ. J. Phys. Chem., 36, 575.
- ORESHKO, V.F., and  
K.A.KOROTCHENKO (1958) : Nauch. Doklady Vysshei Shkoly, Khim i  
Khim Tekhnol, 455. via Chem. Abstr.,  
53, 1795e.
- ORESHKO, V.F. and  
K.A.KOROTCHENKO (1960) : Doklady Akad. Nauk S.S.S.R., 133, 1219.
- PEAT, S., S.J.PIET and  
W.J.WHELAN (1952) : J. Chem. Soc., 705.
- PEAT, S., W.J.WHELAN and  
G.J.THOMAS (1952) : J. Chem. Soc., 4546.
- PERLIN, A.S. (1958) : Canad. J. Chem., 36, 810.
- PHILLIPS, G.O. (1961) : Adv. Carbohydrate Chem., 16, 13.
- PIET, S.J. and W.J.WHELAN  
(1951) : J. Sci. Food Agric., 2, 224.
- POSTERNAK, T. (1935) : Helv. Chim. Acta, 18, 1351.
- POSTERNAK, T. (1951) : J. Biol. Chem., 188, 317.
- POTTER, A.L. and  
W.Z.HASSID (1948) : J. Amer. Chem. Soc., 70, 3488.
- POTTER, A.L., V.SILVEIRA,  
R.M.McCREADY and  
H.S.OWENS (1953) : J. Amer. Chem. Soc., 75, 1335.
- POTTINGER, P.K. and  
I.T.OLIVER (1962) : Biochim. Biophys. Acta, 58, 303.
- RADLEY, J.A. (1953) : "Starch and its Derivatives" Vol. I,  
pages 146-147; Vol. II. Chapman and Hall,  
London.
- RADLEY, J.A. (1960) : Stärke, 12, 201.
- RECONDO, E. and L.F.LELOIR  
(1961) : Biochem. Biophys. Res. Comm., 6, 85.

- SALIMAN, P.M. (1964) : *Analyt. Chem.*, 36, 112.
- SAMEC, M. (1960) : *J. Appl. Polymer Sci.*, 3, 224.
- SAMEC, M. (1961) : *Stärke*, 13, 283.
- SAMEC, M., I. KOSOVINC and  
H. REUSCHL (1958) : *J. Polymer Sci.*, 30, 545.
- SAMOTUS, B. and  
SCHWIMMER (1962) : *Plant Physiol.*, 37, 519.
- SCHACHMAN, H.K. (1959) : "Ultracentrifugation in Biochemistry,"  
Academic Press, New York.
- SCHAER, E. (1896) : *Pharm. Zentralhalle*, 37, 540. via J.A.Radley  
(1953) "Starch and its Derivatives" Vol. I,  
page 237.
- SCHERAGA, H.A. and L.MANDELKERN  
(1953) : *J. Amer. Chem. Soc.*, 75, 179.
- SCHOCH, T.J. (1942) : *J. Amer. Chem. Soc.*, 64, 2954, 2957.
- SCHOCH, T.J. (1945) : *Adv. Carbohydrate Chem.*, 1, 247.
- SCHOCH, T.J. (1962) : *Brewers Digest (U.S.A.)* Feb. issue.
- SCHOCH, T.J. and  
E.C.MAYWALD (1956) : *Analyt. Chem.*, 28, 382.
- SMITH, G.F. (1953) : *Analyt. Chim. Acta*, 8, 397.
- SOMOGYI, M. (1945) : *J. Biol. Chem.*, 160, 61.
- STACEY, M. (1954) : *Adv. Enzymol.*, 15, 301.
- STAUNDINGER, H. (1932) : *Die Hochmolekularen Organischen Verbindungen*, Springer, Berlin.
- STAUNDINGER, H. and  
E. HUSEMANN (1937) : *Annalen*, 527, 195.
- STEPANENKO, B.N. and  
E.M. AFANASYEVA (1957) : *Biochem. (U.S.S.R.)*, 22, 285.
- SVEDBERG, T. and  
K.O. PEDERSEN (1940) : "The Ultracentrifuge," Oxford Univ. Press,  
London.

- TAKOAKA, K., S.KAWAZU, : Hakko Kagaku Zasshi, 38, 88.  
A.NISHIMURA and : via. Chem. Abstr. 55, 4019h.  
A.MISHINA (1960)
- THOMSON, J. (1961) : Ph.D. Thesis, Edinburgh.
- THOMSON, A. and :  
M.L.WOLFROM (1951) : J. Amer. Chem. Soc., 73, 5849.
- UBBELONDE, L. (1937) : Analyt. Chem. 9, 85.
- WALGER, J., J.SZASZI and :  
A.THURANSZKY (1962) : Agrochem es Talajtan, 11, 403. via  
Chem. Abstr., 58, 14438g.
- WALKER, G.J. and :  
W.J.WHELAN (1959) : Nature, 183, 46.
- WHELAN, W.J. (1958) : "Encyclopedia of Plant Physiology," Vol. 6,  
page 154. Springer-Verlag, Berlin.
- WHELAN, W.J. (1963) : Stärke, 15, 247.
- WHELAN, W.J. and J.M.  
BAILEY (1954) : Biochem. J., 58, 560.
- WHELAN, W.J. and S.PEAT :  
(1950) : J. Soc. Dyers and Colourists, 65, 748.  
via. Chem. Abstr., 44, 47001.
- WHISTLER, R.L. and :  
W.M.DOANE (1961) : Cereal Chem., 38, 251.
- WHISTLER, R.L. and :  
G.E.HILBERT (1945) : J. Amer. Chem. Soc., 67, 1161.
- WHISTLER, R.L. and :  
W.L.THORNBURG (1957) : J. Agric. Food Chem., 5, 203.
- WOLF, M.J., M.M.MACMASTERS,  
J.E.HUBBARD and C.E.RIST :  
(1948) : Cereal Chem., 25, 312.
- WOLFF, I.A., B.T.HOFREITER,  
P.R.WATSON, W.L.DEATHERAGE,  
and M.M.MACMASTERS :  
(1955) : J. Amer. Chem. Soc., 77, 1654.
- YOSHIDA, M., M.FUJII,  
J.NIKUNI and B.MARVO :  
(1958) : Mem. Inst. Sci. Ind. Res., Osaka Univ.,  
15, 209.



ZASLOW, B. (1963) : Biopolymers, 1, 165.

ZUBER, M.S., C.O.GROGAN,  
W.L.DEATHERAGE, J.E.HUBBARD,  
W.E.SCHULZE and M.M.MACMASTERS  
(1958) : Agron, J., 50, 9.



An Investigation of the Starch of the Fruit of the Potato, *Solanum tuberosum*\*)

By C. T. GREENWOOD and S. MACKENZIE, Edinburgh (Scotland)

*Introduction*

Some varieties of the potato, *Solanum tuberosum*, tend to produce fruit, or berries, when the plant reaches maturity. In common with many other fruits, the potato berry contains starch. However, this starch has not been investigated before, and we thank Dr. T. J. SCHOCH<sup>1)</sup> for kindly drawing our attention to this problem.

The starch was first featured in a monograph of 1892 by GRIFFITHS (1), who, on microscopic examination, found the granules to possess very prominent concentric lamellations. The only other reference to this starch is a photo-micrograph of these lamellations shown in a recent review by SCHOCH (2).

In view of the possible interest with regard to the problem of biosynthesis, we have now isolated starch granules from the fruit of several varieties of potato in quantities sufficient for detailed investigation. Studies have been made of the properties of the granules and the separated amylose- and amylopectin-components, and these have been compared with those of the corresponding tuber starches.

*Materials*

The samples of potato fruit<sup>2)</sup> were obtained from the Scottish Plant Breeding Station, Roslin, Midlothian, Scotland through the courtesy of Dr. W. BLACK. Three varieties of potato were chosen, primarily for the extent to which they bore fruit; these were two established commercial varieties, i.e. „Dr. MacIntosh“ and „Pentland Dell“, and a Mexican blight-resistant strain of *S. tuberosum*, sub-species *Andigena*. The potato berries

were collected from the plants in late August when they were some 2–2.5 cm. in diameter.

Starch was isolated from the berries by extraction in a blender with 0.01M mercuric chloride; the latter being used to inhibit enzymic activity. The extract was then filtered through muslin, and the granules obtained by sedimentation. They were purified by shaking a saline suspension with toluene as described by BANKS and GREENWOOD (3). After repeated sedimentation through water to remove salt, the granules were stored in aqueous suspension at 0 °C under toluene.

*Experimental**Estimation of the starch-content of the berries*

Portions of the berries were ground with silver-sand and then extracted with cold perchloric acid until the residue gave no stain with iodine. The concentration of starch in the extract was determined colorimetrically by the phenol-sulphuric acid method of SMITH and his coworkers (4).

*Characterization of the granules*

(i) *The protein-content* of the granules was obtained from duplicate semi-micro Kjeldahl determinations.

(ii) *Percentages of phosphorus* in the starch were measured by wet oxidation of the polysaccharide with boiling perchloric acid followed by colorimetric estimation of the phosphomolybdate complex (3).

(iii) *Gelatinization temperatures* were determined using a microscope and electrically-heated microscope stage as described by SCHOCH and MAYWALD (5).

(iv) *Percentages of amylose* were calculated from the results of potentiometric measurements of iodine binding power using a semi-micro differential apparatus (6).

(v) *Granular susceptibility* to attack by 2.2 N hydrochloric acid at 35 °C, was followed using the method of BUTTROSE (7), except that 150 mg. samples were treated with 10 ml. of acid, and drying was carried out in vacuo at 30 °C.

(vi) *Granular susceptibility to amylolytic attack*. Portions of non-dried granules (approx. 500 mg.) were treated with a mixture of barley  $\alpha$ - and  $\beta$ -amylases (pro-

\*) This is Part XXVII in the Series „Physicochemical Studies on Starches“; Part XXVI, Makromol. Chem., in press; Part XXV, Stärke **15** (1963), 166.

<sup>1)</sup> Research Department, Corn Products Company, Argo, Illinois, USA.

<sup>2)</sup> It may be noted that although nearly all varieties of potato produce flowers, pollen-sterility — which is not yet completely understood — restricts the number which actually fruit. However, fruit-bearing potatoes are not popular commercially; the yield of tubers may be adversely affected and the berries very readily seed themselves in the ground.



vided by Mr. A. W. MAC GREGOR) in phosphate buffer (pH 5.5) at 50 °C. The concentration of liberated reducing sugars in the supernatant liquor was then determined colorimetrically at intervals using the phenol-sulphuric acid reagent (4).

#### Fractionation of the granules

Starch granules were dispersed directly into aqueous solution by boiling under reflux for  $\frac{1}{2}$  hr. in a nitrogen atmosphere. Amyloses were precipitated as the thymol-complex, and then recrystallized as the butanol-complex. Amylopectins were obtained by freeze-drying the supernatant liquors after removal of the amylose by high-speed centrifugation. These methods have been described in detail elsewhere (3).

#### Characterization of the amyloses

The extents of conversion of the amylose samples into maltose under (i) the action of pure  $\beta$ -amylase and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme, were measured (3).

Limiting viscosity numbers,  $[\eta]$ , were measured in 1M-potassium hydroxide at 25 °C using a modified Ubbelohde viscometer (3).

#### Characterization of the amylopectins

Measurement of iodine affinity to estimate the purity of the samples, and measurements of the extent of conversion into maltose under the action of pure  $\beta$ -amylase were carried out as above.

Average lengths of unit-chain were calculated from potentiometric determinations of the amount of formic acid liberated on oxidation with sodium metaperiodate at 4 °C (8).

The weight-average molecular weight,  $M_w$ , was determined by lightscattering for some samples in 0.1M sodium chloride. We are indebted to Mr. R. GEDDES for these measurements.

### Results and Discussion

#### Properties of granular starches

The properties of the granular starches from the berry and tuber of the different varieties of potato are shown in Table 1. The potato berry contains some 10 to 13% of starch, which is comparable to that found in immature tubers (9). It has to be stressed that an exact comparison between the berry and tuber starches is not possible. We have found that the characteristics of potato starch granules depend on the maturity, i.e. when the plant matures, there is an increase in the

starch-content of the tuber, whilst there is also a corresponding increase in the size of the granules and the amount of amylose they contain (9). However, general comparisons between the berry and tuber starches can be obviously made. Table 1 shows that the nitrogen- and phosphorus-content of the two types are similar, but the average gelatinization temperatures differ. These differences are shown by the gelatinization curves in Fig. 1. Gelatinization is a complicated pheno-

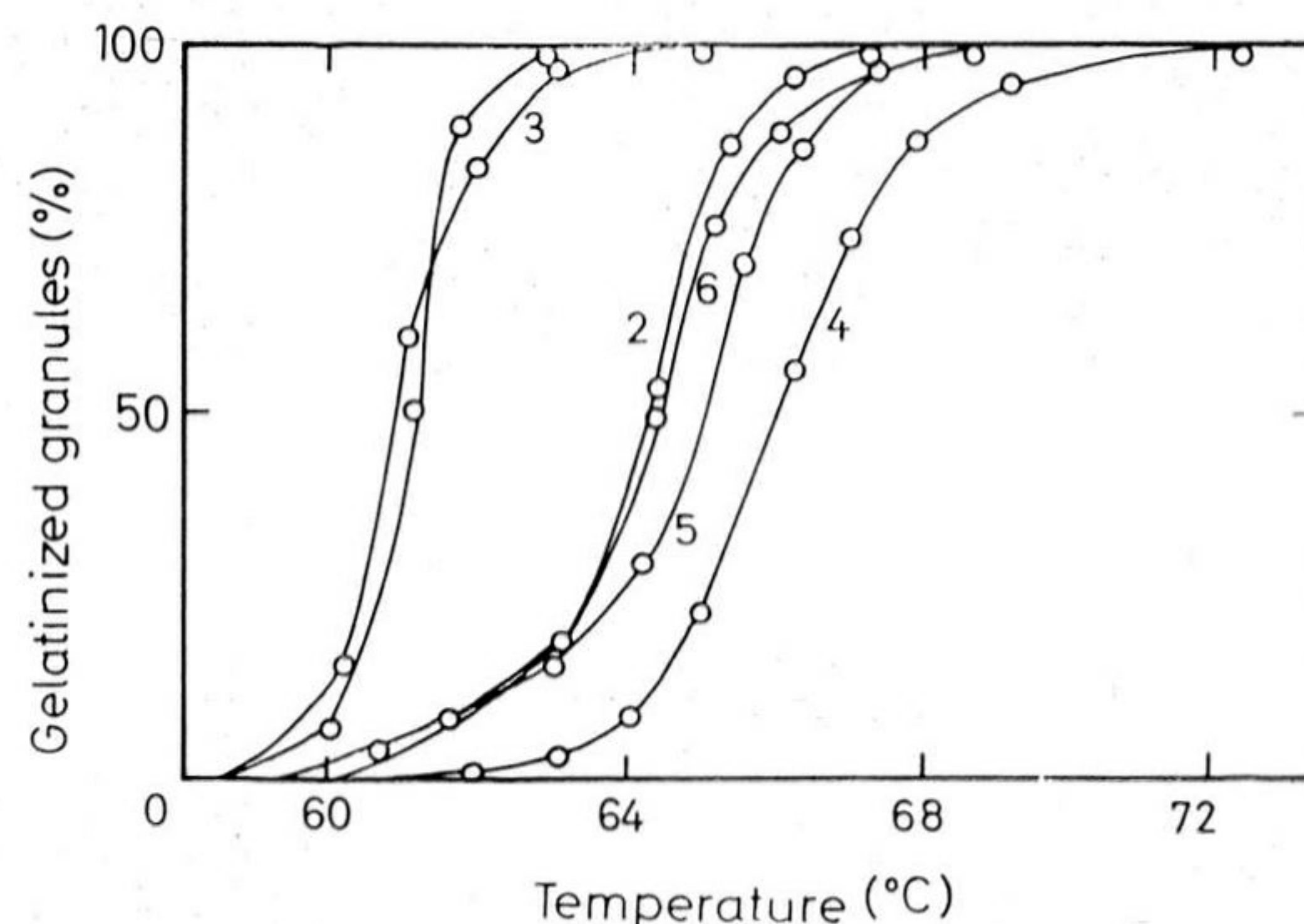


Fig. 1. Percentage of granules gelatinized in water as a function of the temperature:

- |                         |                          |
|-------------------------|--------------------------|
| 1 Dr. MacIntosh, tuber  | 2 Dr. MacIntosh, berry   |
| 3 Pentland Dell, tuber  | 4 Pentland Dell, berry   |
| 5 Mexican strain, tuber | 6 Mexican strain, berry. |

menon depending on the granular size — in any sample, small granules gelatinize less readily than large ones, — the percentage of amylose, and the actual organization, or crystallinity of the granule. Fig. 1 shows that the gelatinization temperatures of two of the berry starches were higher than the corresponding tuber samples. However, microscopic examination indicated that the granules of the tuber starches were significantly smaller. As the amylose-content of all the starches was not radically dis-similar, it would appear that the difference in gelatinization behaviour must be due predominantly to differences in granular size. The relatively small variations in apparent amylose-content found between the different starches were most probably due to differences in maturity (9).

#### Granular structure

The problem of granular structure is complex. Little is yet known of the manner in which the starch components are associated together in the internal structure of the granule, and even external features are not completely understood.

Microscopic examination shows that starch granules possess a layered structure and are composed of a series of concentric shells, which in the case of the potato tuber starch are relatively well-pronounced. In the starch granules from the potato berry, however, the prominence of the lamellar structure is unique as shown in Figs. 2 and 3.

Table 1  
Properties of Starch Granules from the Tuber and Berry of the Potato.

Variety	Starch source	Starch (%)	Nitrogen (%)	Phosphorus (%)	Av. gelatinization temp. (°C)	Iodine affinity <sup>1)</sup>	Amylose (%) <sup>2)</sup>
Dr. MacIntosh	Tuber	—	0.005	0.042	61	4.0	20.5
Dr. MacIntosh	Berry	10	0.007	0.031	64	3.6	18.5
Pentland Dell	Tuber	—	0.004	0.051	61	4.0	20.5
Pentland Dell	Berry	13	0.008	0.054	66	3.8	19.5
Mexican strain	Tuber	—	0.007	0.061	65	3.5	18.0
Mexican strain	Berry	11	0.004	0.042	64	3.4	17.5

<sup>1)</sup> Expressed as mg. I<sub>2</sub> bound per 100 mg. starch

<sup>2)</sup> Calculated from iodine affinity.



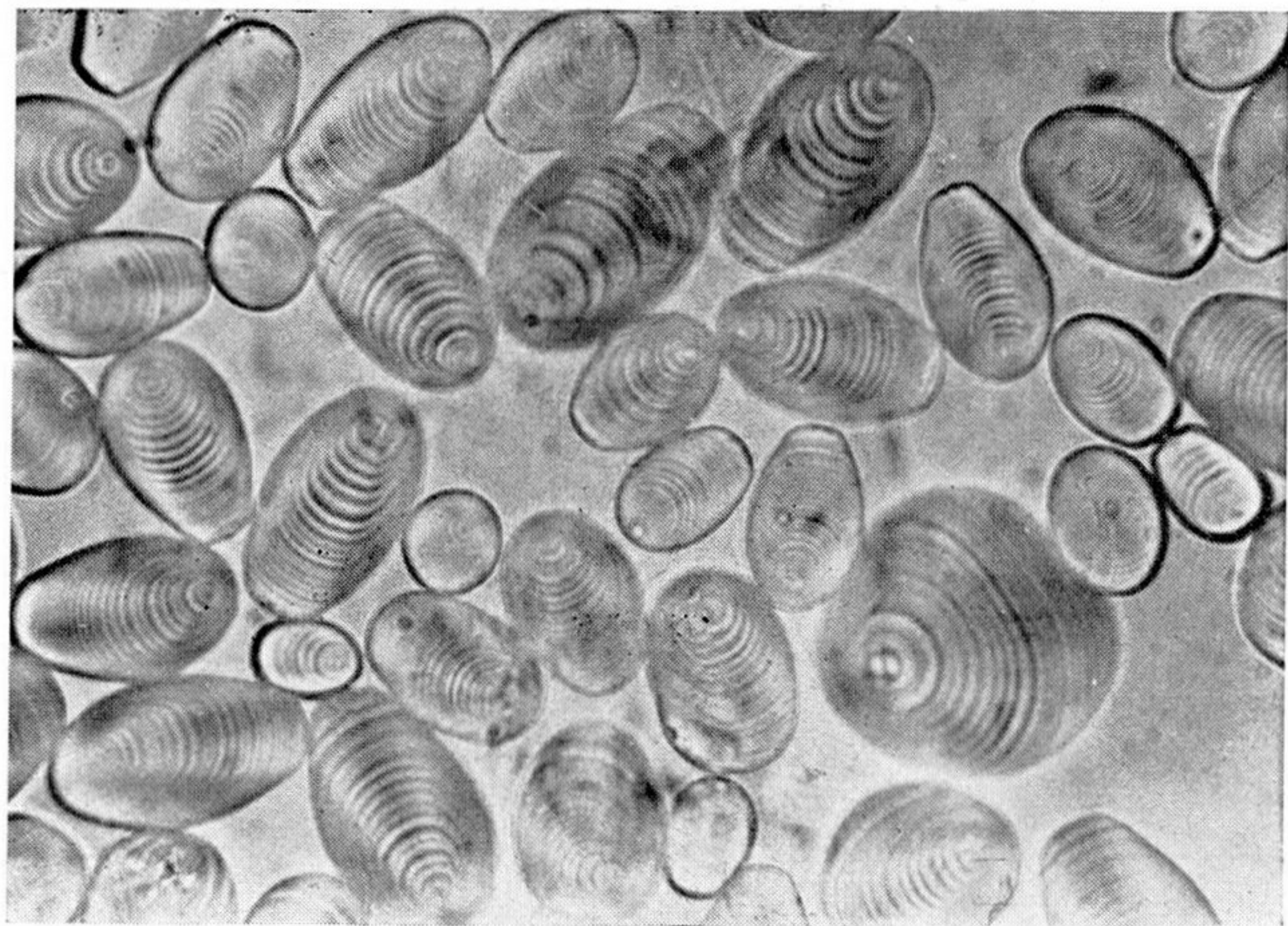


Fig. 2. Photomicrograph of the granules of starch from the potato berry var. Dr. MacIntosh (enlargement,  $\times 1000$ ).

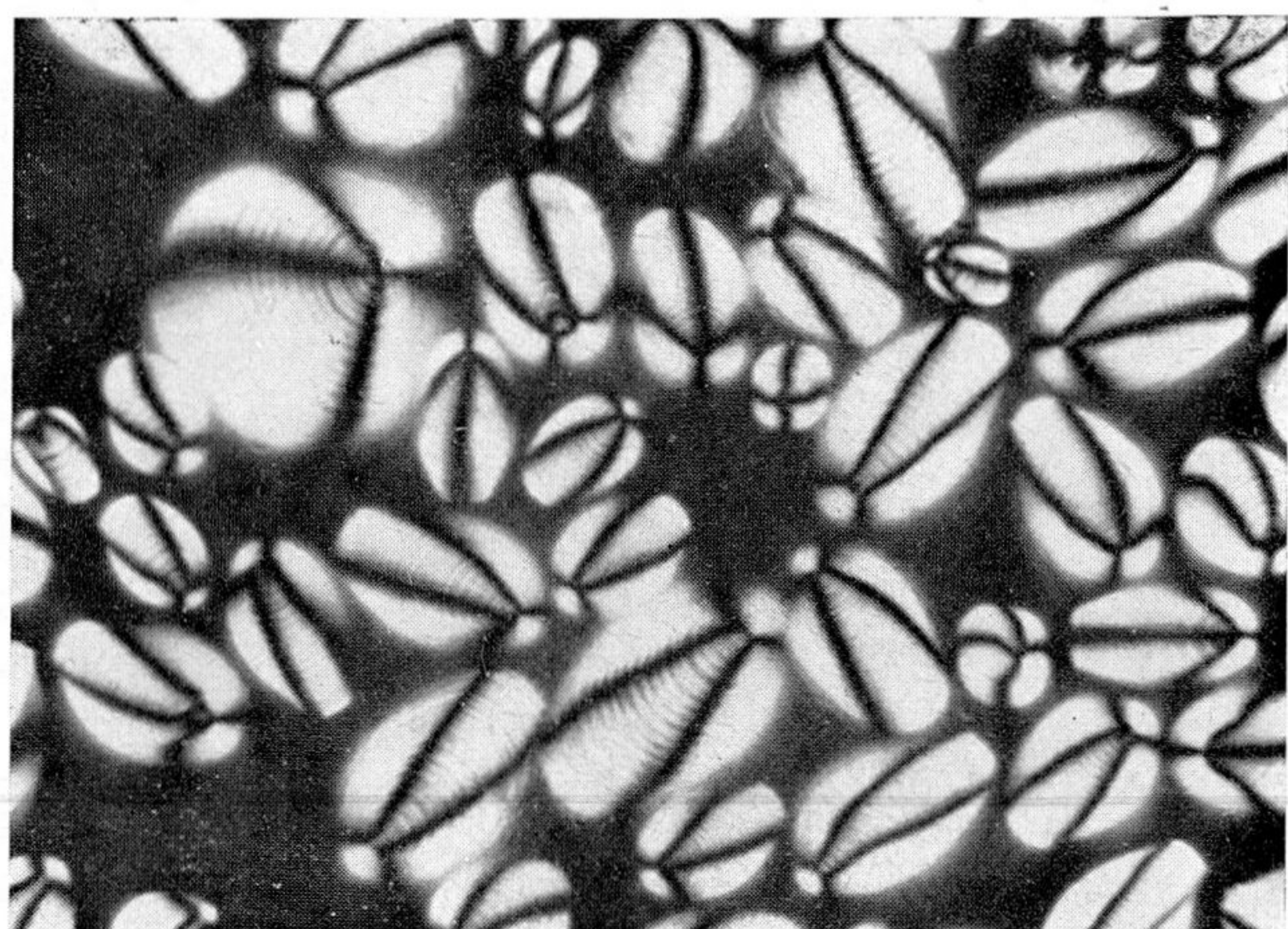


Fig. 3. Photomicrograph of the granules of starch from the potato berry var. Dr. MacIntosh (Polarized light; enlargement  $\times 1000$ ).

The lamellations in potato starches are some  $1-2\mu$  wide and some  $3-4\mu$  apart. Differences in refractive index must cause their appearance, and they may represent some sort of periodic deposition of material during growth by apposition. Early workers suggested in fact, that the shells represented diurnal changes, but recent evidence indicates that this is erroneous. BUTTROSE (10) has found that potato starch grown under constant environment still possessed lamellations. However, barley and wheat starch granules grown under these conditions did not have lamellations. This author therefore suggested that shell formation in potato granules must be controlled by an endogenous rhythm, whilst in the case of the other two starches, it must be controlled by the external environment. Recent electron-microscopy studies (11) on granules, which had been treated with acid, have indicated that these shells consist of some 50–100 submicroscopic lamellae, and the latter may correspond to the actual deposition rhythms.

In an attempt to find out whether the more pronounced lamellations on the berry starches reflected any changes in the organization of the granules, the susceptibility of these starches to attack by acid, and amylolytic enzymes was studied. A typical effect of erro-

sion by 2.2 N hydrochloric acid at  $35^\circ\text{C}$  is shown in Fig. 4. The rate of solubilization of the granules was similar to that found by BUTTROSE (7) for his sample of potato starch. However, no appreciable difference in the rates of erosion between the berry and tuber starches were found. This result is, perhaps, not unexpected, as the ease with which hydrogen ions must be able to permeate the granular structure implies that only gross differences would affect the rate of attack.

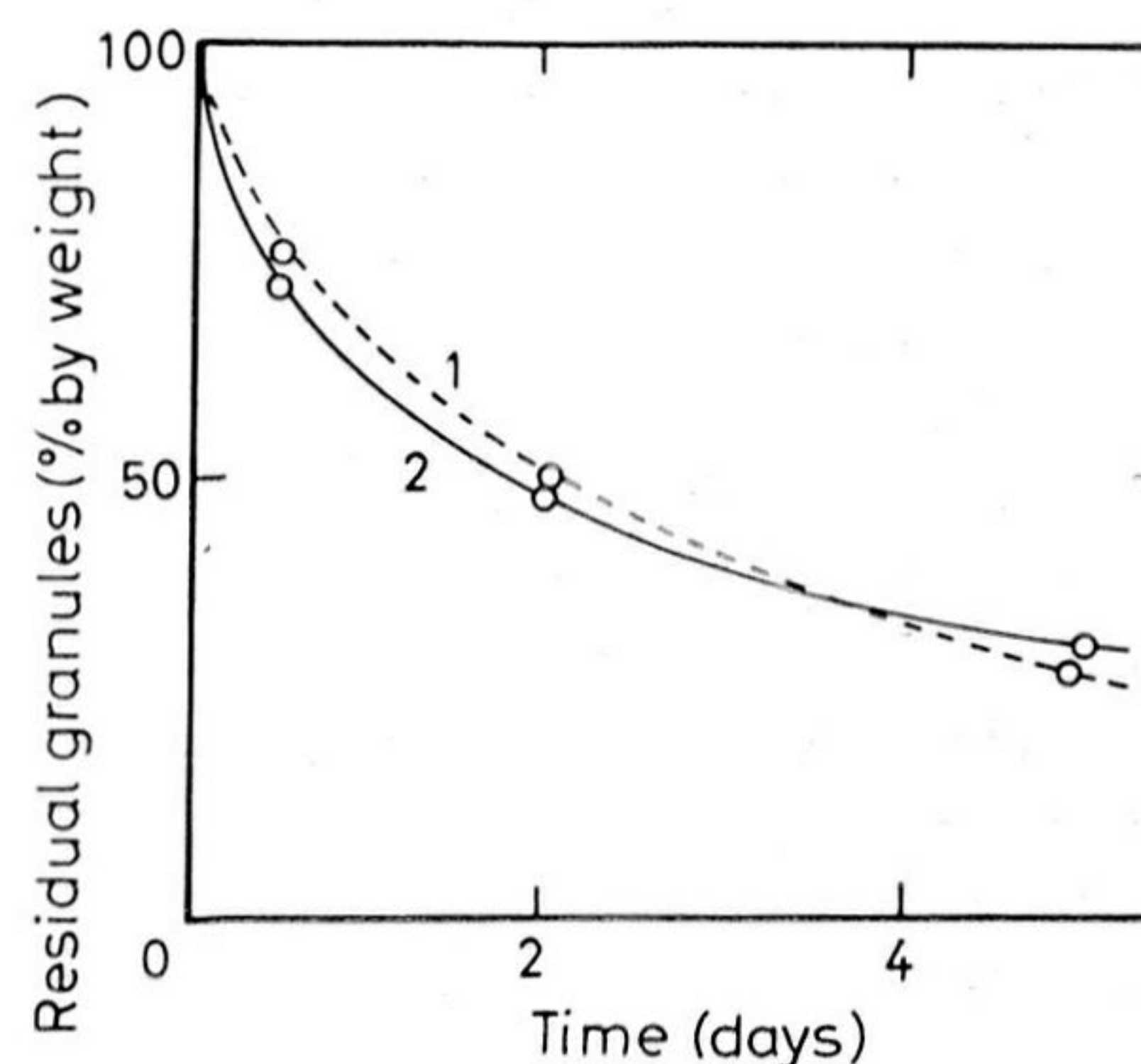


Fig. 4. Percentage residual weight of the granules (Mexican strain) as a function of the time of treatment with 2.2N hydrochloric acid at  $35^\circ\text{C}$ :

1 Berry

2 Tuber

Differences in granular susceptibility became apparent when the granules were treated with a mixture of  $\alpha$ - and  $\beta$ -amylase from barley. Fig. 5 shows that on a qualitative basis the berry starches were more readily attacked.

Microscopically, erosion and enlargement of the hilum was evident in most of the granules, although few of them appeared to be completely destroyed (compare, ref. 12). The iodine affinity of the residue starches after enzymic treatment was substantially

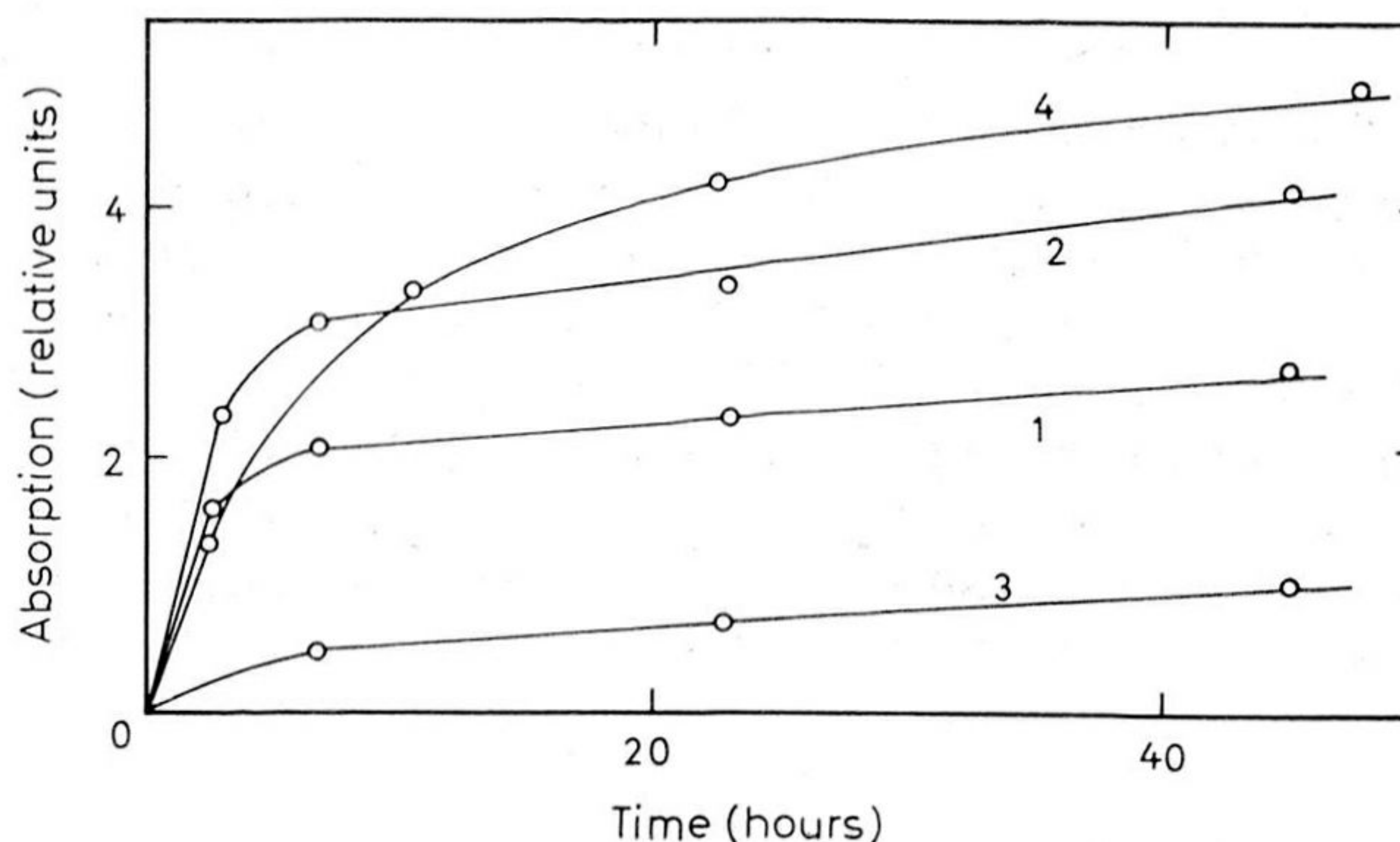


Fig. 5. Amount of liberated saccharides (expressed as relative units from absorption measurements) as a function of the time of treatment of the granules with a mixture of  $\alpha$ - and  $\beta$ -amylase at  $50^\circ\text{C}$ :

1 Dr. MacIntosh, tuber

2 Dr. MacIntosh, berry

3 Mexican strain, tuber

4 Mexican strain, berry.

the same as that for the original material, but it is to be noted that the extent of solubilization under our conditions was limited. Enzymic degradation of starch granules is complicated [compare the comments by



LEACH and SCHOCH (12)], but our results would suggest that there may be a difference — though not a radical one — between the berry and tuber starches.

The above results suggest that the more pronounced lamellations on the berry starch do not cause any obvious differences in physical behaviour of the granules. However, this may be merely a reflection of the current lack of suitable techniques for studying granular organization.

#### Properties of starch components

The properties of the amylose components obtained by fractionating the granular starches are shown in Table 2. Values are given for the percentage conversion

Table 2  
Properties of Amylose Components from Starches.

Variety	Starch source	$\beta$ -Limit (i) <sup>1)</sup>	$\beta$ -Limit (ii) <sup>1)</sup>	$[\eta]$	$\overline{D.P.}$ <sup>2)</sup>
Dr. MacIntosh	Tuber	75	101	540	4000
Dr. MacIntosh	Berry	85	99	450	3300
Pentland Dell	Tuber	85	99	390	2900
Pentland Dell	Berry	88	98	380	2800
Mexican strain	Tuber	80	101	470	3500
Mexican strain	Berry	84	100	420	3100

<sup>1)</sup> Conversion into maltose under the action of (i) pure  $\beta$ -amylase, and (ii)  $\beta$ -amylase and Z-enzyme.

<sup>2)</sup> Degree of polymerization; calculated from  $\overline{D.P.} = 7.4 \times [\eta]$

into maltose under (i) the action of pure  $\beta$ -amylase, and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme. [Z-enzyme is a common contaminant of  $\beta$ -amylase preparations, and has been shown to have properties similar to  $\alpha$ -amylase (13)]. For all samples, degradation by the pure  $\beta$ -amylase was incomplete, although no contaminating amylopectin was present as shown by the 100% conversion into maltose on treatment with the mixture of enzymes. [Under the experimental conditions used, amylopectin is unaffected by the Z-enzyme (13)]. These samples of amylose must all contain, therefore, a „barrier“ to the action of  $\beta$ -amylase, as would appear to be the general case for samples from a variety of sources (8). Values for the degree of polymerization shown in Table 2 are calculated from the observed limiting viscosity numbers measured in 1 M-potassium hydroxide by a relation obtained earlier (14). The re-

sults are not thought to be exact, but should be of the correct order of magnitude. The values for the berry-amyloses were smaller than those for the corresponding tuber-samples. However, this may only reflect differences in maturity, as other results have shown that the size of the amylose depends on maturity (9).

The purity,  $\beta$ -amylolysis limit, average length of unit-chain and calculated internal chain-length for the amylopectins are shown in Table 3. There are no significant differences in the value of these properties for the samples. The weight-average molecular weights,  $M_w$ , for some of the samples were measured by lightscattering. Fig. 6 shows a typical ZIMM-plot obtained by Mr. GEDDES (these results will be described elsewhere).

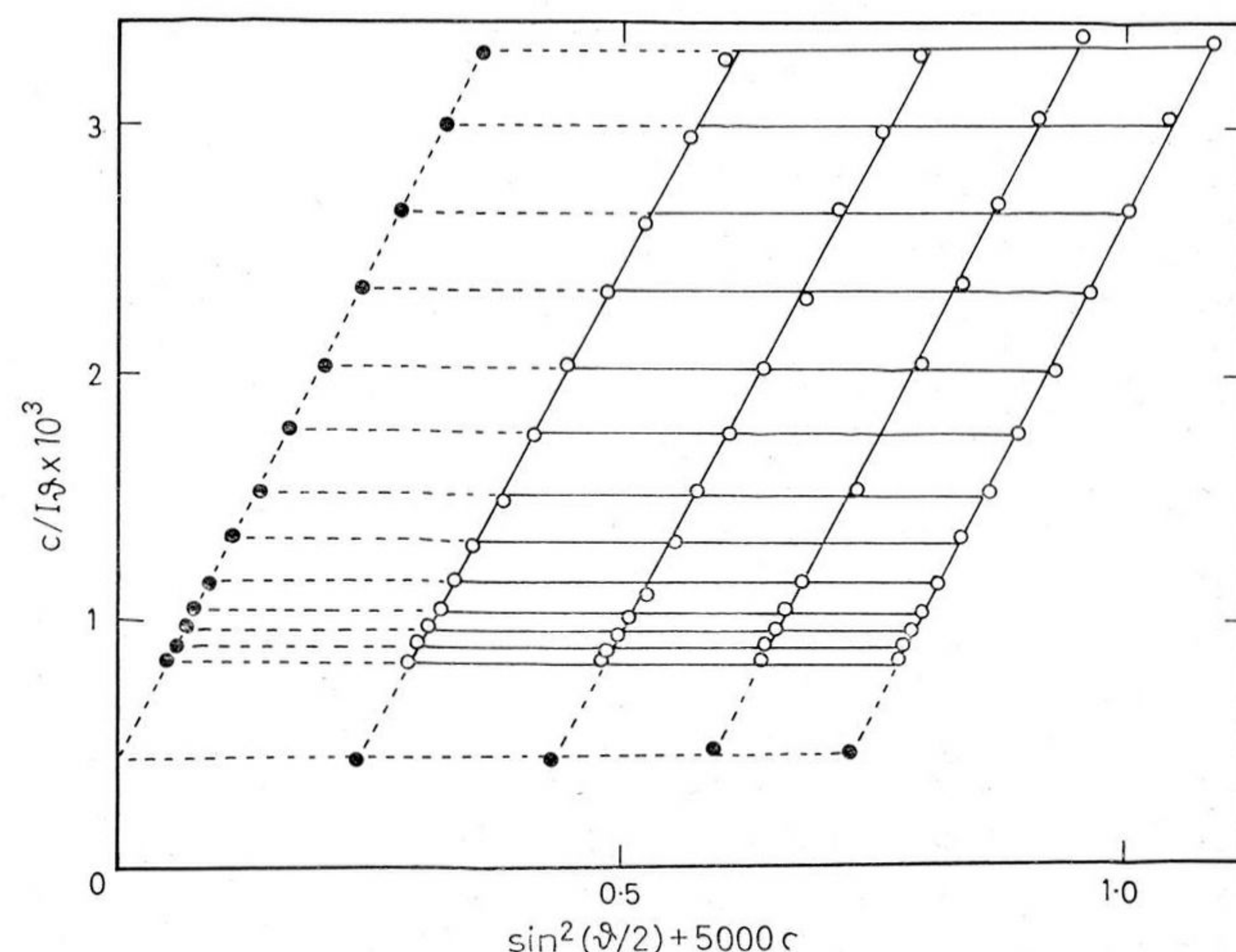


Fig. 6. Typical lightscattering results; ZIMM-plot for tuber-amylopectin (var. Dr. MacIntosh) in 0.1 M-NaCl.

The values obtained for the molecular weight and the radius of gyration (shown in Table 3) are of the same order as we have found in our earlier work; the variation is probably due to differences in maturity.

In general, therefore, the properties of the components from the berry- and tuber-starches are comparable; a radical change in the method of biosynthesis of starch in the berry compared to that in the tuber would appear to be unlikely.

#### Acknowledgements

We wish to thank Miss Tarrant for taking the photomicrographs, and the Corn Industries Research Foundation (Washington, D.C., USA) for a Grant which supported this work.

#### Summary

1. Starch granules have been isolated from the berries of three varieties of the potato plant, *Solanum tuberosum*, in quantities sufficient for complete characterization.

2. The properties of the berry starches have been compared with those of the starch from the corresponding tuber. Differences in nitrogen-content, phosphorus-content, gelatinization tempera-

Table 3  
Properties of Amylopectin Components from Starches.

Variety	Starch source	Purity (%) <sup>1)</sup>	$\beta$ -Limit <sup>2)</sup>	Av. chain-length	Length of internal chain <sup>3)</sup>	Molecular weight ( $\times 10^{-6}$ ) <sup>4)</sup>	Radius of gyration ( $\text{\AA}$ ) <sup>4)</sup>
Dr. Mac Intosh	Tuber	> 99	58	25	8	110	2280
Dr. MacIntosh	Berry	> 99	57	24	8	55	1810
Pentland Dell	Tuber	> 99	58	24	8	8	1210
Pentland Dell	Berry	> 99	58	24	8	35	1650
Mexican strain	Tuber	> 99	57	26	9	—	—
Mexican strain	Berry	> 99	58	26	9	—	—

<sup>1)</sup> From results of potentiometric iodine titrations.

<sup>2)</sup> Conversion into maltose under the action of pure  $\beta$ -amylase.

<sup>3)</sup> Calculated from chain-length-[(chain-length  $\times \beta$ -limit) + 2.5].

<sup>4)</sup> From lightscattering measurements.



ture and iodine affinity of the two types of starch were not profound.

3. Microscopic examination showed the potato berry starches to possess very prominent concentric lamellations. The significance of these is discussed.

4. The resistance of the granules to acidic- and enzymic-attack was studied. The berry and tuber starches were degraded to similar extents by acid, but on contact with amylolytic enzymes, the berry starches were found to be eroded more easily.

5. The granules were fractionated into amylose and amylopectin, and the purity,  $\beta$ -amylolysis limits, and molecular size of the components were measured. All the amylose samples were incompletely degraded into maltose by  $\beta$ -amylase, whilst the berry-amyloses were smaller in molecular size than the corresponding tuber-samples. The  $\beta$ -amylolysis limits, and average-lengths of unit-chain of all the amylopectin samples were similar. The weight-average molecular weight of some of the amylopectins has been measured by lightscattering.

### Zusammenfassung

1. In zu einer vollständigen Charakterisierung ausreichenden Mengen wurden Stärkekörner aus den Beeren drei verschiedener Arten der Kartoffelpflanze, *Solanum tuberosum*, isoliert.

2. Die Eigenschaften der Beerenstärken wurden mit denen der Stärke entsprechender Knollen verglichen. Abweichungen im Gehalt an Stickstoff und Phosphor, Verkleisterungstemperatur und Jod-Affinität der beiden Stärkearten sind unbedeutend.

3. Die mikroskopische Untersuchung ergab, daß Kartoffelbeerenstärken hervorragende konzentrische Schichtung besitzen, über deren Bedeutung gegenwärtig noch diskutiert wird.

4. Die Säure- und Enzymbeständigkeit der Körner wurde untersucht. Beeren- und Kartoffelknollenstärken wurden in ähnlichem Umfang mit Säure abgebaut; es zeigte sich jedoch, daß die Beerenstärken in Berührung mit amylolytischen Enzymen leichter angegriffen werden.

5. Die Körner wurden in Amylose und Amylopektin fraktioniert, und Reinheit, beta-Amylosebereiche und Molekülgröße der Bestandteile gemessen. Alle Amylosemuster wurden unvollständig durch beta-Amylose in Maltose abgebaut, wobei die Beerenamylosen eine kleinere Molekülgröße aufwiesen als die entsprechenden Knollenmuster. Beta-Amylolysebereiche und Durchschnittslänge aller Amylopektinmuster waren ähnlich. Das mittlere Molekulargewicht einiger der Amylopektine wurde durch Lichtstreuung gemessen.

### Résumé

1. Des quantités suffisantes de grains d'amidon permettant une caractérisation complète furent isolées des baies de trois différentes sortes de la plante de pommes de terre „*solanum tuberosum*“.

2. Les propriétés des amidons des baies furent comparées avec celles des amidons des tubercules correspon-

dants. Les différences de la teneur en azote et en phosphore, de la température de glutination et de l'affinité vis à vis de l'iode des deux sortes d'amidons sont sans importance.

3. Les recherches microscopiques ont montré que les amidons des baies de pommes de terre possèdent des couches concentriques excellentes sur l'importance desquelles nous discuterons encore.

4. On a analysé la résistance des grains envers les acides et les enzymes. Les amidons des baies et des tubercules de pommes de terre ont été décomposés dans la même mesure à l'aide d'acides. Les résultats ont pourtant montré que les amidons des baies sont attaqués plus facilement par les enzymes amylolytiques.

5. Les grains ont été fractionnés en amylose et en amylopectine et on a mesuré les zones de la  $\beta$ -amylose et de la grandeur moléculaire des composants. Tous les échantillons d'amylose ont été décomposés en maltose à l'aide de  $\beta$ -amylase. Les amyloses des baies présentaient une grandeur moléculaire plus petite que les échantillons de tubercules correspondants. Les zones de la  $\beta$ -amylose et la longueur moyenne de tous les échantillons d'amylopectine étaient pareilles. Le poids moléculaire moyen de différentes amylopectines fut déterminé à l'aide de la dispersion lumineuse.

### References

- (1) GRIFFITHS, W.: „The Principal Starches used as Food“, Baily and Son, Cirencester, England, **1892**, pp. 59—60.
- (2) SCHOCH, T. J.: *Brewers Digest* (USA), Feb. 1962 issue.
- (3) BANKS, W., and C. T. GREENWOOD: *Biochem. J.* [London] **73** (1959), 237.
- (4) DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH: *Analytic. Chem.* **28** (1956), 350.
- (5) SCHOCH, T. J., and E. C. MAYWALD: *Analytic. Chem.* **28** (1956), 382.
- (6) ANDERSON, D. M. W., and C. T. GREENWOOD: *J. chem. Soc.* [London] (1955), 3016.
- (7) BUTTROSE, M. S.: *Stärke* **15** (1963), 85.
- (8) GREENWOOD, C. T., and J. THOMSON: *J. chem. Soc.* [London] (1962), 222.
- (9) GREENWOOD, C. T., and S. MACKENZIE: unpublished observations.
- (10) BUTTROSE, M. S.: *J. Cell Biol.* **14** (1962), 159.
- (11) FREY-WYSSLING, A., and M. S. BUTTROSE: *Makromol. Chem.* **44—46** (1961), 173.
- (12) LEACH, H. W., and T. J. SCHOCH: *Cereal Chem.* **38** (1961), 34.
- (13) BANKS, W., C. T. GREENWOOD and I. G. JONES: *J. chem. Soc.* [London] (1960), 150.
- (14) COWIE, J. M. G., and C. T. GREENWOOD: *J. chem. Soc.* [London] (1957), 2862.

Address of the Authors: C. T. Greenwood, D. Sc., and S. MacKenzie, B. Sc., Department of Chemistry, The University, Edinburgh 9 (Scotland).

(Arrived: 10. 6. 1963)



**Sonderdruck**

**aus der Fachzeitschrift „Die Stärke“**

**Jahrgang 15, Heft 12, Seite 444-448**

**1963**



## The Irradiation of Starch. Part I

### The Properties of Potato Starch and its Components after Irradiation with High-Energy Electrons\*)

By C. T. GREENWOOD and S. MACKENZIE, Edinburgh (Scotland)

#### *Introduction*

Recently, the effect of various radiations on the structure and properties of starch has been studied. The irradiation has been carried out by X-rays (1),  $\gamma$ -rays (1–16), neutrons (3), high-energy electrons (14, 17), ultrasonics (14, 18) and ultra-violet rays (18). In the majority of instances, granular starch has been investigated, and it would appear that all such radiations modify the granular structure. Degradative effects are caused primarily, i.e. breakdown of the molecular chains, as shown by the changes observed in properties such as viscosity, gelatinization, and alkaline lability. At the same time, small fission-products — dextrins, sugars, sugar acids and volatile gases ( $\text{CO}$ ,  $\text{CO}_2$  and  $\text{CH}_4$ ) — may also be formed. Similar degradative phenomena occur when gels, or dispersions, of starch are investigated (19, 20, 21, 22, 23), or when the

fractionated starch components are irradiated in either the solid state (15, 24), or solution (23, 25).

RADLEY (17) has shown that high-energy electrons, in doses of up to  $10^8$  rads. in total, on maize starch, alter (a) physical properties, such as viscosity and gelatinization behaviour, and (b) chemical properties such as iodine-stain, reducing-power, and amount of carboxyl-groups. Through the courtesy of Mr. J. A. RADLEY<sup>1)</sup>, we are now able to report studies of the properties of potato starches treated with smaller total doses of this radiation (max.  $10^7$  rads.). Furthermore, the effect of the radiation on the properties of the amylose and amylopectin components in the granule has been investigated.

#### *Materials*

The samples of commercial potato starch obtained from Mr. J. A. RADLEY<sup>1)</sup> had been degassed and sealed

\*) This is Part XXX in the Series, „Physicochemical Studies on Starches“; Part XXIX, *Stärke* **15** (1963), 359.

<sup>1)</sup> The J. A. RADLEY Research Institute, 220—222 Elgar Road, Reading, Berkshire (England).



in polythene bags in an atmosphere of nitrogen. They had then been irradiated with high energy electrons from a 2-meV VAN DER GRAAF generator. Doses ranged from  $5 \times 10^4$  to  $10 \times 10^6$  rads. During irradiation the temperature did not rise appreciably. This procedure has been detailed by RADLEY (17).

### Experimental

#### Characterization of the granules

(i) Acidity of the samples was determined by potentiometric titration of a suspension of starch (1 g in 10 ml of water; in a nitrogen atmosphere) with 0.01 M sodium hydroxide.

(ii) Gelatinization temperatures were determined using a microscope and electrically-heated microscope stage as described by SCHOCH and MAYWALD (26).

(iii) The iodine affinity of the starches was determined by potentiometric measurements using a semi-micro, differential apparatus (27).

#### Fractionation of the starches

The method of fractionation differed slightly from our usual procedure (28); in view of the acidity of the samples, they were dispersed in boiling water containing phosphate buffer ( $p_H$  6.4; 4 ml per 1 l of water). Amylose was precipitated first with thymol, and then recrystallized with butanol. The amylopectin-containing supernatant liquor was extracted with ether, concentrated on a rotatory-evaporator (temperature  $< 35^\circ\text{C}$ ), and the resultant solution dialysed against distilled water, prior to freeze-drying.

#### Characterization of the amyloses

The extents of conversion of the amylose samples into maltose were measured for (i) the action of pure  $\beta$ -amylase, and (ii) the concurrent action of  $\beta$ -amylase and Z-enzyme (28). Limiting viscosity numbers,  $[\eta]$ , in 1 M-potassium hydroxide were determined at  $25^\circ\text{C}$ .

#### Characterization of the amylopectins

The iodine affinity of the samples, and the extent of conversion into maltose under the action of pure  $\beta$ -amylase were measured as above.

The optical density of solutions of the amylopectins (5 mg/100 ml) stained with iodine (0.0020% iodine + 0.020% potassium iodide/100 ml.) were measured in 1 cm cells in a spectrophotometer.

Average-lengths of unit-chain were calculated from potentiometric determinations of the amount of formic acid liberated on oxidation with sodium metaperiodate at  $4^\circ\text{C}$  (29).

Sedimentation measurements were made on aqueous solutions using a BECKMAN-SPINCO model E Ultracentrifuge.

### Results and Discussion

#### Properties of the granular starches

The acidity of the starches, shown in Table 1, was appreciably less than that found by RADLEY (17) for samples which had received higher doses of high-energy electrons. There was no obvious damage to the granular structure under microscopic observation, and all starches exhibited birefringent properties. Furthermore, the behaviour on gelatinization and the gelatinization temperatures were not altered appreciably, except for

Table 1  
Properties of Irradiated Granular Starches

Sample	Irradiation dose (rads. $\cdot 10^{-6}$ )	Acidity (equivs./g. $\cdot 10^6$ )	Gelatinization temperature ( $^\circ\text{C}$ )	Iodine affinity <sup>1)</sup>	Amylose (%) <sup>2)</sup>
A	0	4.4	68—69	4.5	23
B	0.05	6.0	—	4.4	22.5
C	0.1	7.4	67—69	4.3	22
D	0.2	8.5	—	4.1	21
E	0.5	10.3	67—69	3.6	18.5
F	1	12.7	—	3.4	17.5
G	2	17.9	64—65	3.1	16
H	5	29.0	—	2.4	12
I	10	40.0	60—61	1.1	6

<sup>1)</sup> Expressed as mg. of  $\text{I}_2$  bound per 100 mg of starch. Derived from Fig. 1, as described in Text.

<sup>2)</sup> Apparent amylose-content. Calculated from iodine affinity.

sample I. However, gelatinization is not a sensitive measure of granular modification, for quite profound differences were apparent when the iodine-binding power of the samples was measured. The results of these experiments are shown in Fig. 1. It can be seen that as the radiation-dose increases, there is a lowering in iodine binding-power of the starch. In fact, the changes induced by the high-energy electrons in samples B-D, appear to be identical to those we have reported for the acid-degradation of potato starch granules (30).

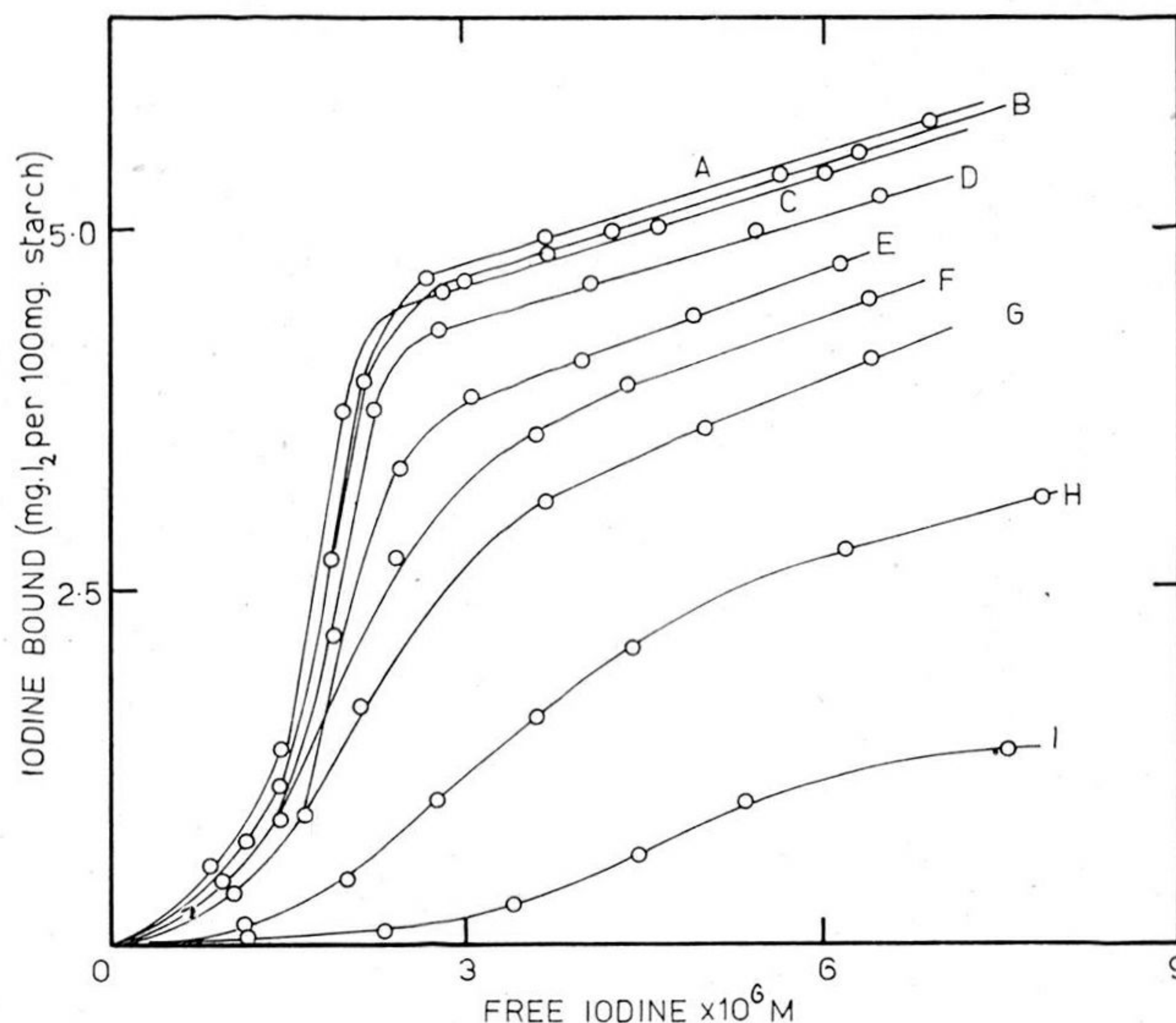


Fig. 1. Potentiometric iodine titration results for starches; letters on curves correspond to samples in Table 1.

For samples E-I, the modification of the starch is so profound that the usual sigmoidal adsorption isotherm is not found, and, furthermore, the concentration of free iodine necessary to saturate the sample increases. Our usual procedure (27) of extrapolating the linear portion of the adsorption curve to zero free iodine concentration, in order to obtain the iodine affinity, is not applicable in these cases. An approximate value for the iodine affinity was obtained, therefore, from the point of intersection of the rising, binding position of the curves and the linear adsorption curve. The resultant values are shown in Table 1, although those for samples H and



I are approximate only. Apparent values for the amylose-content shown in the last column of Table 1 were arbitrarily calculated from:  $\% \text{ amylose} = [(\text{iodine affinity}) / 19.5] \times 100$ . The shape of the iodine-binding curves for samples E-I indicated a radical loss

of iodinebinding material, and suggested that the amylose component was degraded in these starches.

Through the courtesy of Mr. D. J. BRYCE, the irradiated starches were pyrolysed in vacuo at 300°C, and the decomposition products examined by gas-chromatography (31). The chromatograms obtained were found to be identical to those for non-irradiated starches, and so the presence of any small molecular weight fission-products did not alter appreciably the pyrolysis „cracking-pattern“ of the polymer.

#### Properties of the components

The properties of material, which was precipitated with conventional precipitating-agents for amylose, is shown in Table 2. The enzymic experiments showed

Table 2

Properties of Amylose Components from Starches

Sample <sup>1)</sup>	$\beta$ -Limit (i) <sup>2)</sup>	$\beta$ -Limit (ii) <sup>2)</sup>	$[\eta]$	D. P. <sup>3)</sup>
A	83	100	230	1700
B	86	102	220	1650
C	88	99	150	1100
D	88	98	110	800
E	87	92	95	700
F	86	91	80	600
G	84	89	50	350
H	83	86	40	300
I	83	87	35	250

<sup>1)</sup> As in Table 1

<sup>2)</sup> Conversion into maltose under the action of (i) pure  $\beta$ -amylase, and (ii)  $\beta$ -amylase and Z-enzyme.

<sup>3)</sup> Number-average degree of polymerization; calculated from  $\overline{D.P.} = 7.4 \cdot [\eta]$ .

that only samples B-D of the irradiated starches contained „amylose“ in the sense that they were incompletely degraded by pure  $\beta$ -amylase (i.e. contained some type of structural modification), while being completely degraded with the mixture of  $\beta$ -amylase and Z-enzyme (i.e. contained no amylopectin). The radiation did, in fact, introduce modifications into the  $\alpha$ -1:4-glucan structure, as the extent of degradation by  $\beta$ -amylase was reduced. For samples E-I, although the  $\beta$ -amylolysis limits altered, some other type of structural modification had occurred, as the polysaccharide was incompletely degraded by the mixture of  $\beta$ -amylase and Z-enzyme. It is not thought likely that this could be contaminating, degraded amylopectin-material.

The decrease in value of the limiting viscosity number,  $[\eta]$ , for the amylose samples, as shown in Table 2, indicated that extensive depolymerization was occur-

Table 3

Optical Density<sup>1)</sup> of Iodine-complex of Some Amylopectins

Sample	Wavelength (m $\mu$ )											
	460	480	500	520	540	560	580	600	620	640	660	680
A	0.217	0.248	0.268	0.304	0.332	0.333	0.316	0.287	0.255	0.235	0.193	0.164
E	0.191	0.219	0.251	0.288	0.314	0.319	0.297	0.265	0.235	0.200	0.184	0.150
I	0.252	0.280	0.319	0.356	0.376	0.370	0.334	0.293	0.255	0.215	0.180	0.130

<sup>1)</sup> For conditions, see Text

ring with increasing doses of irradiation. The extent of this is shown by curve 1 in Fig. 2. Approximate values of the degree of polymerization,  $\overline{D.P.}$ , of the samples are given in the last column of Table 2. The relation between the very low values of  $\overline{D.P.}$  for samples G-I, and the  $(\beta + Z)$ -limit discussed above, is being further investigated.

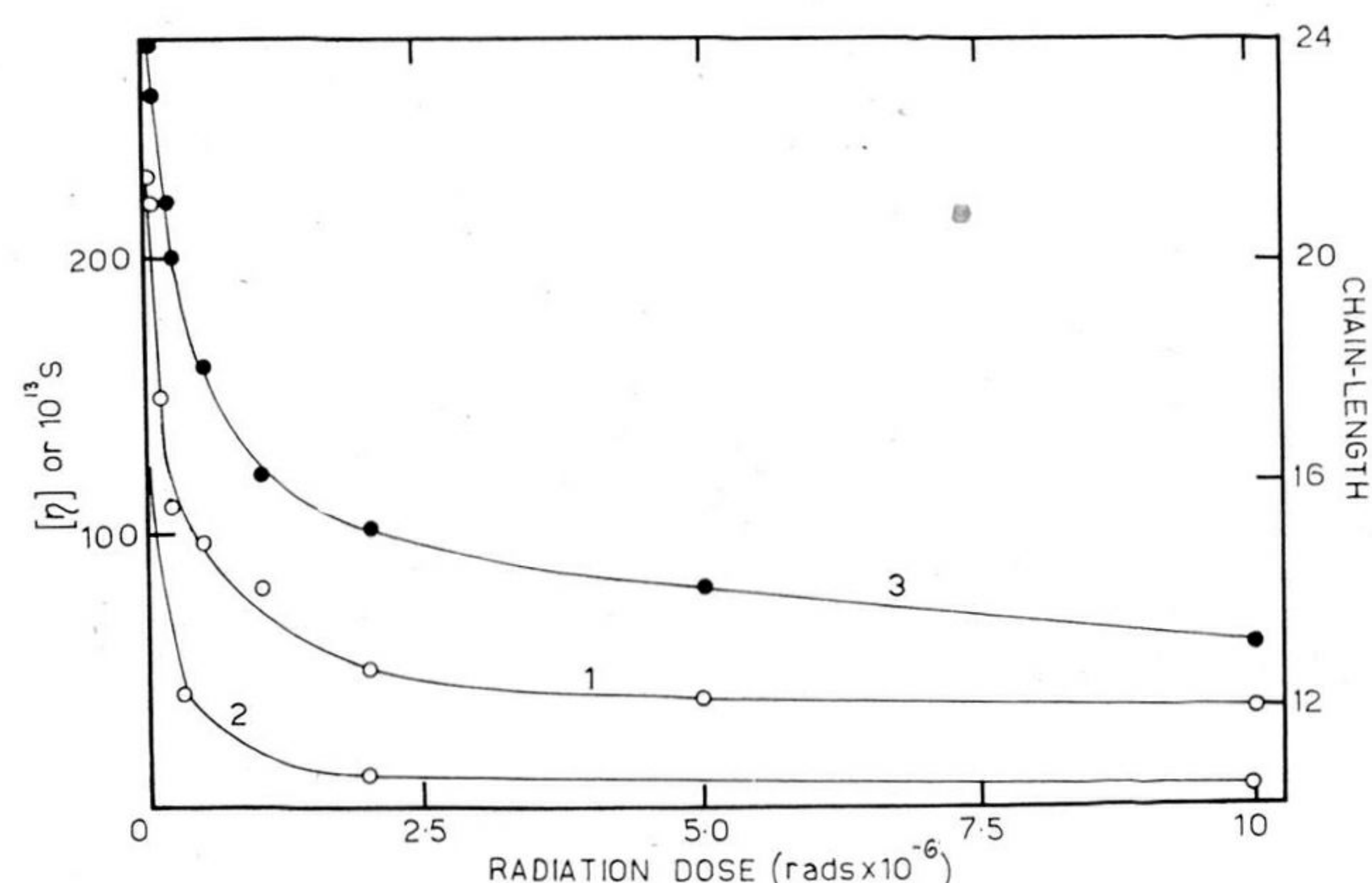


Fig. 2. Effect of radiation dose on the properties of the starch components. Variation of:

1. limiting viscosity number,  $[\eta]$ , for amylose.
2. sedimentation coefficient,  $S$ , for amylopectin
3. chain-length (in glucose units) for amylopectin

In the case of the amylopectin samples, the method of isolation would necessitate that they would contain any soluble, degraded, amylose-type material. However, there was no evidence for this. The iodine binding-power of the amylopectins from irradiated samples was much less, in fact, than that for the control-sample, A, as shown by the typical results in Fig. 3. This is in contrast to the behaviour of amylopectins from acid-degraded starch (30). Again, the optical density of io-

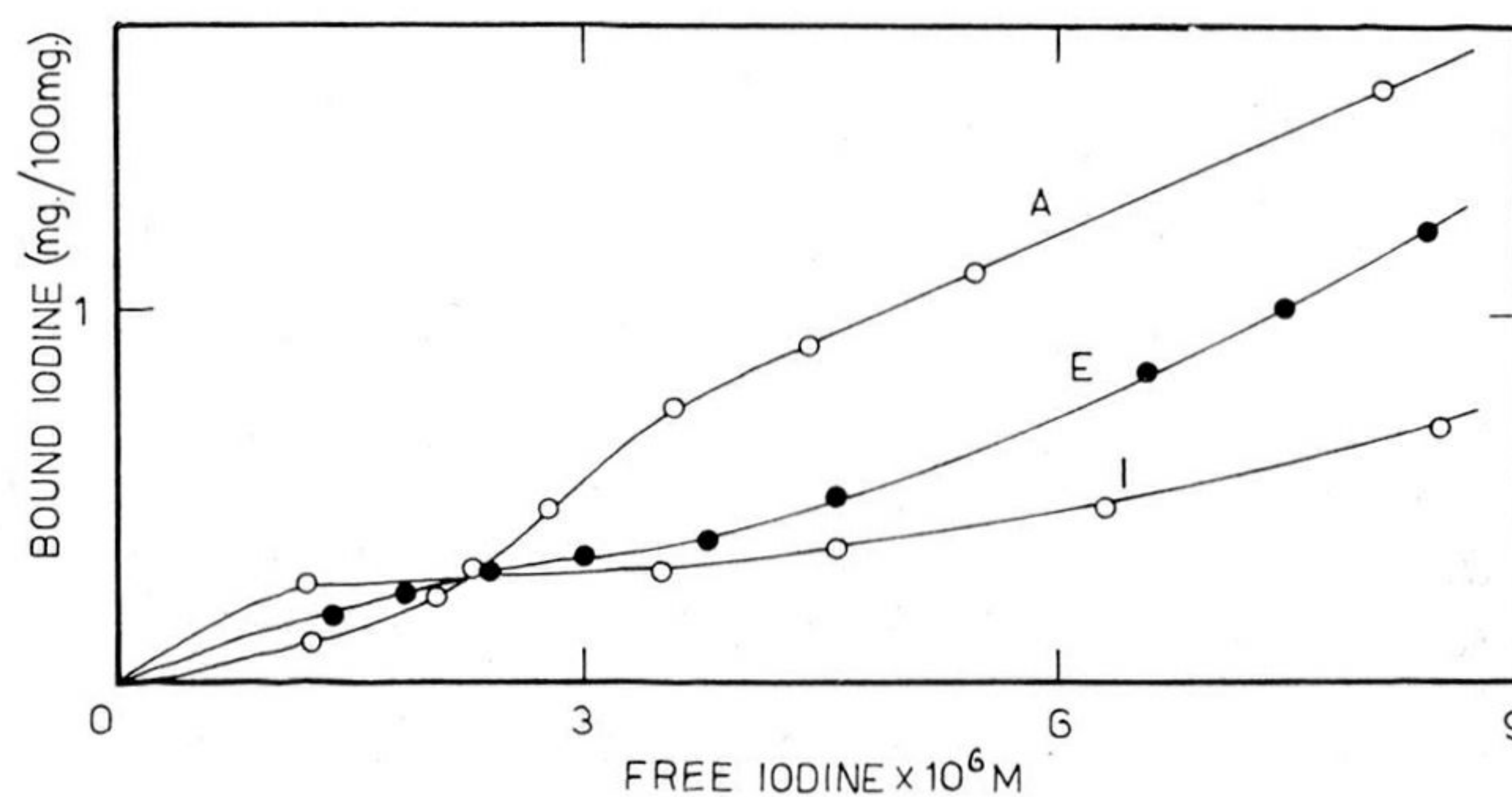


Fig. 3. Typical potentiometric iodine titration results for amylopectins; letters on curves correspond to samples in Table 1.



dine-complexes showed a maximum absorption at about 550 m $\mu$ , with no evidence of any peak at higher wavelengths corresponding to linear material. Typical results are shown in Table 3.

Profound depolymerization and structural changes were apparent in the amylopectin components. The samples were very readily soluble in cold water, indicating a reduction in molecular size. This was confirmed by ultracentrifuge measurements, when the sedimentation coefficient was found to decrease rapidly with irradiation dose, as shown in curve 2 in Fig. 2. Simultaneously, as well as depolymerization of the main chain, breaks must also have occurred in the branches, as indicated by changes in the average-length of unit-chain shown in Table 4. The extent of this, in relation

Table 4

Properties of Amylopectin Components from Starches

Sample <sup>1)</sup>	$\beta$ -Limit <sup>2)</sup>	Av. chain-length	Length of internal-chain <sup>3)</sup>
A	58	24	8
B	57	23	7
C	55	23	7
D	54	21	7
E	51	18	6
F	49	16	6
G	49	15	5
H	48	14	5
I	46	13	5

<sup>1)</sup> As in Table 1

<sup>2)</sup> Conversion into maltose under the action of pure  $\beta$ -amylase

<sup>3)</sup> Calculated from {chain-length — [(chain-length  $\cdot$   $\beta$ -limit) + 2.5]}.

to the radiation-dose, is shown by curve 3 in Fig. 2.  $\beta$ -Amylolysis limits, shown in Table 4, indicate that the irradiated polysaccharide is less amenable to enzymic degradation. This may again indicate the formation of structural modifications.

### Conclusions

It is apparent that, although there is relatively little change in microscopic-appearance, or gelatinization temperature, on irradiation of starch granules with high-energy electrons in total doses of up to  $10^7$  rads., quite profound depolymerization of the starch components occurs at doses as low as  $5 \times 10^4$  rads. The breakdown of the polymeric chains may be accompanied by structural modifications. These phenomena are being investigated further.

### Acknowledgements

We thank Mr. J. A. RADLEY, M. Sc., F.R.I.C., F.S.D.C., for providing the irradiated starch samples, and the Corn Industries Research Foundation (Washington, D.C., USA) for a Grant, which supported this work.

### Summary

1. The acidity, gelatinization temperature, and iodine affinity have been measured for potato starch samples, which had been irradiated with high-energy

electrons at total doses of from  $0.05 \times 10^6 - 10 \times 10^6$  rads.

2. With increase in radiation dose, although the gelatinization behaviour was little affected, changes occurred in the iodine affinity, and the apparent amylose-content of the starch decreased.

3. The irradiated starches have been fractionated into their amylose and amylopectin components, and the properties of these have been compared with those for the non-irradiated control sample.

4. For all of the irradiated starches, the amylose and amylopectin components showed evidence of gross molecular degradation from the results of viscosity and sedimentation measurements. This depolymerization was accompanied by structural modifications, as indicated by changes in susceptibility to degradation by  $\beta$ -amylase and Z-enzyme.

### Zusammenfassung

1. An Kartoffelstärken, die mit beschleunigten Elektronen mit einer Gesamtdosis von  $0,05 \times 10^6 - 10^6$  rad bestrahlt wurden, sind Säuregrad, Verkleisterungstemperatur und Jodbindevermögen gemessen worden.
2. Eine Erhöhung der Bestrahlungsdosis wirkte sich nur geringfügig auf das Verkleisterungsverhalten aus, jedoch ergaben sich Veränderungen des Jodbindevermögens, und der scheinbare Amylosegehalt der Stärke nahm ab.
3. Die bestrahlten Stärken wurden in ihre Amylose- und Amylopektinbestandteile fraktioniert, deren Eigenschaften mit denen einer nicht bestrahlten Kontrollprobe verglichen wurden.
4. Bei allen bestrahlten Stärkeproben zeigten die Amylose- und die Amylopektinbestandteile einen starken molekularen Abbau, wie aus den Messungen der Viskosität und der Sedimentbildung hervorging. Neben der Depolymerisation traten auch Strukturumwandlungen auf, wie aus den Veränderungen der Angreifbarkeit durch  $\beta$ -Amylase und Z-Enzym ersichtlich ist.

### Résumé

1. On a mesuré le degré d'acidité, la température de glutination et le pouvoir de liaison envers l'iode de différents amidons de pommes de terre, qui avaient été soumis à des électrons accélérés dont la dose totale était de  $0,05 \times 10^6 - 10^6$  rad.
2. Une augmentation de la dose de radiation n'agissait que faiblement sur le pouvoir de glutination, mais on a pu observer des changements pour le pouvoir de liaison envers l'iode. La teneur apparente en amylose de l'amidon était diminuée.
3. On a fractionné les amidons irradiés dans leurs composants d'amylose et d'amylopectine dont on a comparé les propriétés à celles d'un échantillon de contrôle non irradié.
4. Chez tous les échantillons irradiés l'amylose et l'amylopectine montraient une forte dégradation moléculaire, comme on l'a pu constater à partir de mesures de la viscosité et de la formation de sédiments. A côté d'une dépolymérisation on a pu enregistrer des modifications structurelles qui furent démontrées par des changements quant à l'attaque par la  $\beta$ -amylase et l'enzyme-z.



## References

- (1) EHRENBERG, L., M. JAARMA and K. G. ZIMMER: *Acta chem. scand.* **11** (1957), 950.
- (2) SAMEC, M.: *Stärke* **13** (1961), 105, and refs. therein.
- (3) SAMEC, M.: *Stärke* **15** (1963), 83.
- (4) KHENOKH, M. A.: *Doklady Akad. Nauk, S.S.S.R.* **104** (1955), 746; via *Amer. chem. Abs.* **50** (1955), 7603 b.
- (5) KERTESZ, Z. I., E. R. SCHULTZ, G. FOX and M. GIBSON: *Food Res.* **24** (1959), 609.
- (6) MISHINA, A., and Z. NIKUNI: *Nature [London]* **184** (1959), 1867.
- (7) ORESHKO, V. F., and K. A. KOROTCHENKO: *Doklady Akad. Nauk, S.S.S.R.* **133** (1960), 1219; via *Amer. chem. Abs.* **54** (1960), 23385 h.
- (8) SAMEC, M.: *J. appl. Polymer Sci.* **3** (1960), 224.
- (9) ORESHKO, V. F., L. F. GORIN and G. M. MASLOVA: *Izvest Vysshikh Ucheb. Zavedenii, Pishchevaya Tekhnol.* (1961), 35; via *Amer. chem. Abs.* **56** (1962), 617 c.
- (10) ORESHKO, V. F., L. E. CHERENKO and N. G. SHAKHOVA: *Doklady Akad. Nauk, S.S.S.R.* **134** (1960), 636; via *Amer. chem. Abs.* **55** (1961), 13976 i.
- (11) ORESHKO, V. F., *Zhur. Fiz. Khim.* **34** (1960), 2369; via *Amer. chem. Abs.* **55** (1961), 11046 d.
- (12) TAKAOKA, K., S. KAWAZU, A. NISHIMURA and A. MISHINA: *Hakko Kogaku Zasshi* **38** (1960), 88; via *Amer. chem. Abs.* **55** (1961), 4019 h.
- (13) SAMEC, M., and S. JENSIC: *Hoppe-Seyler's Z. physiol. Chem.* **322** (1960), 1.
- (14) SAMEC, M.: *Stärke* **13** (1961), 283.
- (15) DOGUCHI, M., Y. YOKOYAMA and I. OKADA: *Kogyo Kagaku Zasshi* **64** (1961), 2001; via *Amer. chem. Abs.* **57** (1963), 7503 b.
- (16) KHENOKH, M. A., E. A. KUZICHEVA and V. F. EVDOKIMOV: *Doklady Akad. Nauk, S.S.S.R.* **135** (1960), 471; via *Amer. chem. Abs.* **55** (1961), 11316 e.
- (17) RADLEY, J. A.: *Stärke* **12** (1960), 201.
- (18) SAMEC, M., I. KOSOVING and H. REUSCHL: *J. Polymer Sci.* **30** (1958), 545.
- (19) KHENOKH, M. A.: *Zhur. Obshchei. Khim.* **20** (1950), 1560; via *Amer. chem. Abs.* **45** (1951), 963 d.
- (20) KHENOKH, M. A.: *Trudy Pervogo Vsesoyuz. Soveshchaniya po Radiatsion. Khim. Akad. Nauk S.S.S.R.* (1957), 188; via *Amer. chem. Abs.* **53** (1959), 12020 a.
- (21) ORESHKO, V. F., and K. A. KOROTCHENKO: *Nauch. Doklady Vysshei Shkoly, Khim. i Khim. Tekhnol.* (1958), 455; via *Amer. chem. Abs.* **53** (1959), 1795 e.
- (22) ORESHKO, V. F., L. E. CHERENKO and N. G. SHAKHOVA: *Doklady Akad. Nauk, S.S.S.R.* **134** (1960), 363; via *Amer. chem. Abs.* **55** (1961), 13976 i.
- (23) WHELAN, W. J., and S. PEAT: *J. Soc. Dyers Colourists* **65** (1950), 748.
- (24) DILLI, S., and J. L. GARNETT: *Chem. and Ind. [London]* (1963), 409.
- (25) BOURNE, E. J., M. STACEY and G. VAUGHAN: *Chem. and Ind. [London]* (1956), ~~189~~ **543**.
- (26) SCHOCH, T. J., and E. C. MAYWALD: *Analytic. Chem.* **28** (1956), 382.
- (27) ANDERSON, D. M. W., and C. T. GREENWOOD: *J. chem. Soc. [London]* (1955), 3016.
- (28) BANKS, W., and C. T. GREENWOOD: *Biochem. J. [London]* **73** (1959), 237.
- (29) GREENWOOD, C. T., and J. THOMSON: *J. chem. Soc. [London]* (1962), 222.
- (30) COWIE, J. M. G., and C. T. GREENWOOD: *J. chem. Soc. [London]* (1957), 2658.
- (31) BRYCE, D. J., and C. T. GREENWOOD: *Stärke* **15** (1963), 166, 285.

*Address of the Authors: C. T. Greenwood, D. Sc., and S. MacKenzie, B. Sc., Department of Chemistry, The University, Edinburgh 9 (Scotland)*

*(Arrived: 26. 8. 1963)*

Die STÄRKE erscheint monatlich. Bestellungen nimmt jede Buchhandlung des In- und Auslandes, die Post oder der Verlag entgegen. In den Ländern Belgien, Dänemark, Großbritannien, Italien, Luxemburg, Niederlande, Norwegen, Portugal, Schweden, der Schweiz und der Vatikanstadt ist der Bezug durch die Post ebenfalls möglich. *Bezugspreis:* vierteljährlich DM 12.—, Einzelheft DM 5.—.

**Probeheft: kostenlos durch den Verlag.**

Wissenschaftliche Verlagsgesellschaft m.b.H., Stuttgart N, Birkenwaldstraße 44, Postfach 40.